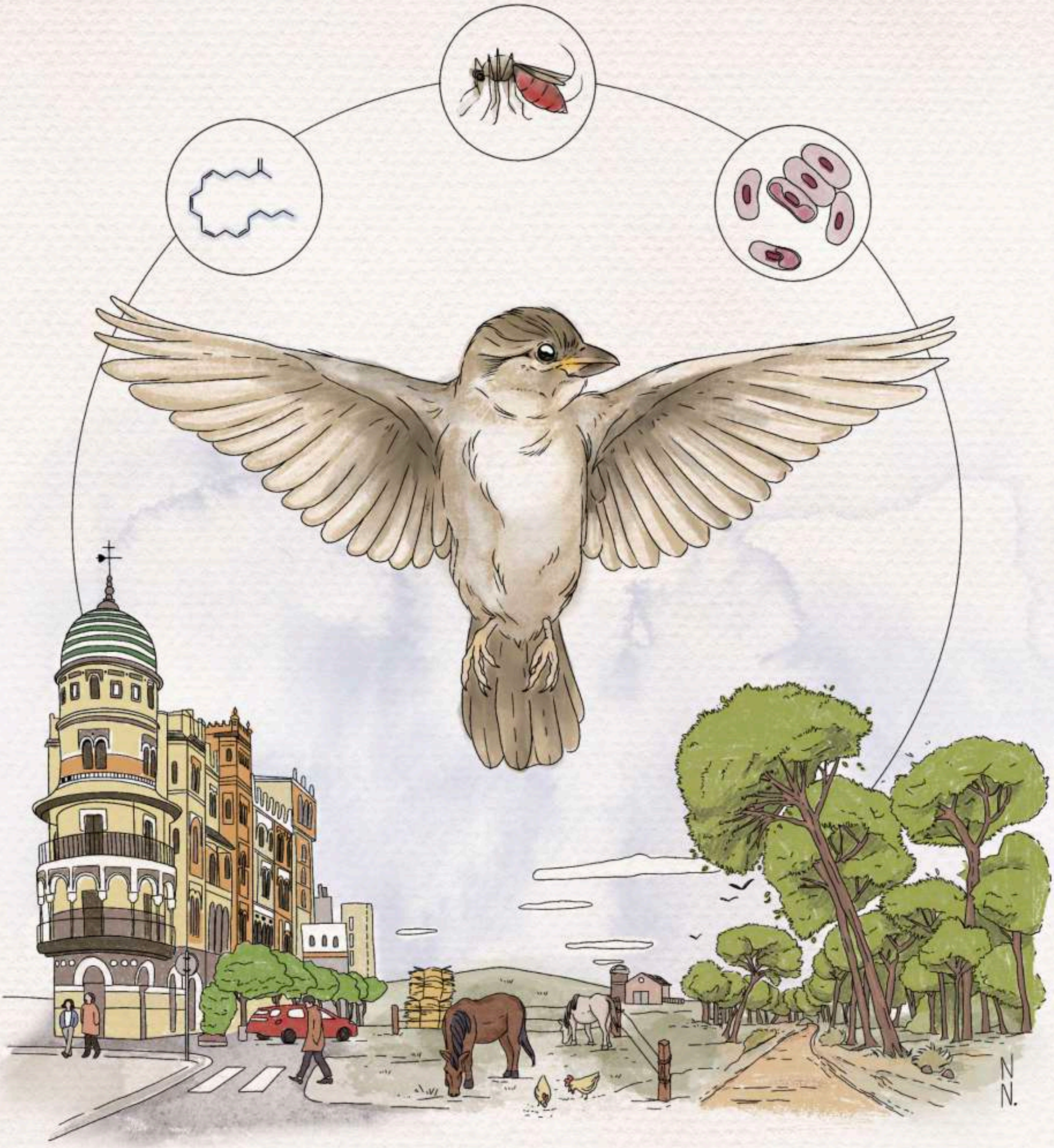
IMPACT OF LANDSCAPE ANTHROPIZATION ON PATHOGEN TRANSMISSION DYNAMICS AND WILD BIRDS HEALTH



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Jéssica Jiménez Peñuela

PhD Thesis

Seville, 2021

Estación Biológica de Doñana (EBD-CSIC)
Departamento de Ecología de Humedales

Universidad de Sevilla
Facultad de Biología
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Impact of landscape anthropization on pathogen transmission dynamics and wild birds health

Memoria presentada por la Graduada Jéssica Jiménez Peñuela para optar al
título de Doctora por la Universidad de Sevilla

JéssicaJP

Fdo. Jéssica Jiménez Peñuela

Dr. **Jordi Figuerola Borras**, Investigador Científico de la Estación Biológica de Doñana (EBD-CSIC); Dr. **Josué Martínez de la Puente**, Profesor Ayudante Doctor de la Universidad de Granada (UGR); Dra. **Martina Ferraguti**, Investigadora Postdoctoral de la University of Amsterdam (UA) como director, co-director y co-directora, y **Javier Balbontín Arenas**, Investigador de la Universidad de Sevilla (US) como tutor académico CERTIFICAN:

Que los trabajos de investigación desarrollados en la Memoria de Tesis Doctoral "*Impact of landscape anthropization on pathogen transmission dynamics and wild birds health*", son aptos para ser presentados por la Graduada Jéssica Jiménez Peñuela ante el Tribunal que en su día se designe, para aspirar al grado de Doctora por la Universidad de Sevilla. Y para que así conste, y en cumplimiento de las disposiciones legales vigentes, firman el presente documento en Sevilla, a 23 de Julio de 2021.

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“Happiness can be found, even in the darkest of times,

if one only remembers to turn on the light”

“It is our choices that show what we truly are,

far more than our abilities”

“Words are our most inexhaustible source of magic,

capable of both inflicting injury and remedying it”

– Albus Percival Wulfric Brian Dumbledor –

A mi iaio,

A mi familia,

A mis Figuerólidos

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ABSTRACT

The intensification of agriculture, animal husbandry and the expansion of cities, has led to changes in environmental conditions, reduced biodiversity and altered interactions between organisms affecting many ecological processes, involving pathogen transmission. In addition, detrimental effects of habitat anthropization on the life history of many species of birds have been reported, including in some species apparently thriving in urban areas. Urbanization affects not only the life history, abundance, and composition of vertebrate communities, but also those of hematophagous insects living in these areas and, consequently, the vector-borne pathogens they transmit. Urbanization can alter the incidence and distribution of vector-borne parasites, as well as the susceptibility of vertebrates to infectious diseases through its effects on host immunocompetence.

In this thesis, I used a multidisciplinary approach to determine how the dynamics of vector-borne parasites are affected by habitat anthropization, and how the joint effects of anthropization and parasite infections impact the health of wild birds. For this purpose, I focussed on the widespread vector-borne parasites of the genus *Plasmodium*, commonly known as avian malaria parasites, and the related genera *Haemoproteus* and *Leucocytozoon*, which were molecularly identified. In addition, I used the house sparrow (*Passer domesticus*), one of the best examples of urban exploiter historically associated with human settlements, as the avian study model species. Since the 1970s, this bird species has been suffering a dramatic decline across Europe, especially in cities, with human activities and infectious diseases being identified as the main factors.

Using an extensive dataset of birds sampled at localities in southern Spain, I first determined how vector-borne parasites dynamics are affected by habitat anthropization (**Section 1**). To do that, I compared the variation in the prevalence and richness of the three genera of blood parasites over two years in 15 wild house sparrow populations that included urban, rural, and natural habitats (**Ch. 1**). The prevalence of both *Haemoproteus* and *Leucocytozoon* parasites differed and were correlated between years, while no significant differences were found for *Plasmodium* parasites. Variation in the environmental requirements between vector groups, habitat characteristics and climatic variability strongly determined the stability and activity of vector populations, potentially affecting bird exposure and thus prevalence in the population. Both *Haemoproteus* and *Leucocytozoon* richness also varied with habitat and time. Variation in the composition of vector and/or bird communities may affect the number and identity of parasite lineages circulating and infecting house sparrows, but host-related factors and susceptibility may also explain the infection patterns found.

Secondly, I determined the impact of both anthropization and parasite infections on the health of wild house sparrows (**Section 2**). In the three chapters included in this section, I measured three major variables reflecting the health of birds, namely body condition (**Ch. 2**), the composition of plasma fatty acids (**Ch. 3**) and the oxidative status (**Ch. 4**) in relation to *Plasmodium*, *Haemoproteus* and *Leucocytozoon* infection status and including house sparrow populations from urban, rural and natural habitats. Results show that urbanization increased the levels of fatty acids and oxidative damage to lipids and negatively affected bird body condition which, in turn, influenced the activity of antioxidant enzymes. Among other possibilities, this could be due to the low availability of good quality food in urban habitats that prevent birds to fulfil physiological requirements during development resulting in lower body condition, immunocompetence and antioxidant capacity. Moreover, *Plasmodium* infected juvenile house sparrows had lower proportion of ω-6 and higher proportion of ω-3 PUFAs, as well as lower activity of antioxidant enzymes (GPx, SOD and GR). These results may reflect that, due to the low antioxidant activity of infected birds, only birds with moderate levels of pro-inflammatory fatty acids against parasite infection were likely to survive.

Additionally, results from this thesis show that birds exposed to two different sources of stress, namely urbanization and parasite infection, may suffer greater deleterious effects. Birds from natural habitats infected by *Plasmodium* showed lower ω-6/ω-3 ratio and tended to have a higher proportion of ω-3. It is likely that, due to differential availability of ω-3 PUFA between habitats, only infected house sparrows from natural habitats can promote a greater anti-inflammatory immune response to *Plasmodium* infections because the consumption of food rich in ω-3 PUFA. Moreover, higher body condition of juvenile house sparrows was associated with infections by *Plasmodium* and *Haemoproteus*, especially in urban habitats. This result may be explained by a higher mortality rate of infected birds with worst body condition unable to respond to infection, consequently, infected individuals surviving to infection were in a higher body condition than uninfected ones.

To sum up, in this thesis I provide novel information on the dynamics of vector-borne parasites in anthropized ecosystems and the joint negative effects of urbanization and parasite infections on wild birds. I highlight the importance of also including habitats with different landscape uses, such as rural habitats, in the studies of urbanization impact, where the effect of human activities and domestic animals on wildlife may differ from cities. Moreover, pollutants levels, availability of green spaces or the stability of resources may widely differ among cities, which may determine the final effects on wildlife health and the interpretation that researchers can make of the impact of urbanization. This work contributes to the growing area of *Urban Ecology* and may help to better understand the effects that both urbanization and pathogens have on the ecology and evolution of wildlife under the current scenario of global change.

Resumen

La intensificación de la agricultura, la ganadería y la expansión de las ciudades ha provocado cambios en las condiciones ambientales, reducido la biodiversidad y alterado las interacciones entre organismos afectando a muchos procesos ecológicos, incluyendo la transmisión de enfermedades. Además, se han detectado efectos perjudiciales de la antropización del hábitat en la historia de vida de diferentes especies de aves, incluyendo especies que aparentemente prosperan en áreas urbanas. La urbanización no solo afecta la historia de vida, abundancia y composición de las comunidades de vertebrados, sino también de los insectos hematófagos que viven en estas áreas y, en consecuencia, los patógenos transmitidos por estos vectores. La urbanización puede alterar la incidencia y distribución de parásitos transmitidos por vectores, así como la susceptibilidad de los vertebrados a enfermedades infecciosas a través de sus efectos sobre la inmunocompetencia del huésped.

En esta tesis, utilicé un enfoque multidisciplinario para determinar cómo la dinámica de los parásitos transmitidos por vectores se ve afectada por la antropización del hábitat, y cómo los efectos conjuntos de la antropización y las infecciones por parásitos impactan en la salud de las aves silvestres. Con este propósito, me centro en los parásitos transmitidos por vectores del género *Plasmodium*, comúnmente conocidos como parásitos de la malaria aviar, y los géneros relacionados *Haemoproteus* y *Leucocytozoon*, que fueron identificados molecularmente. Además, utilicé el gorrión común (*Passer domesticus*), uno de los mejores ejemplos de ave urbanita asociado históricamente con los asentamientos humanos como ave modelo de estudio. Desde la década de 1970, esta especie está experimentando un descenso dramático en toda Europa, especialmente en ciudades, siendo las actividades humanas y las enfermedades infecciosas señaladas como factores principales.

Utilizando un extenso conjunto de datos de aves muestreadas en localidades del sur de España, primero determiné cómo la antropización del hábitat afecta la dinámica de los parásitos transmitidos por vectores (**Sección 1**).

Para ello, comparé la variación de la prevalencia y la riqueza de los tres géneros de parásitos sanguíneos durante dos años en 15 poblaciones de gorriones silvestres que incluyeron hábitats urbanos, rurales y naturales (**Cap. 1**). La prevalencia de los parásitos *Haemoproteus* y *Leucocytozoon* difirió y a su vez se correlacionó entre años, mientras que no se encontraron diferencias significativas para los parásitos *Plasmodium*. La variación en los requisitos ambientales entre grupos de vectores, las características del hábitat y la variabilidad climática determinaron fuertemente la estabilidad y la actividad de las poblaciones de vectores, potencialmente afectando la exposición de las aves y, por lo tanto, la prevalencia de parásitos en la población. La riqueza de *Haemoproteus* y *Leucocytozoon* también varió con el hábitat y el tiempo. La variación de la composición de las comunidades de vectores y/o aves puede afectar el número y la identidad de los linajes de parásitos que circulan e infectan al gorrión común, pero los factores relacionados con el hospedador y su susceptibilidad también pueden explicar los patrones de infecciones encontrados.

En segundo lugar, determiné el impacto de la antropización y las infecciones por parásitos en la salud de los gorriones salvajes (**Sección 2**). En los tres capítulos incluidos en esta sección, medí tres variables principales que reflejan la salud de las aves, incluyendo la condición corporal (**Cap. 2**), la composición de los ácidos grasos en el plasma (**Cap. 3**) y el estado oxidativo (**Cap. 4**) en relación con el estado de infección por *Plasmodium*, *Haemoproteus* y *Leucocytozoon* e incluyendo poblaciones de gorrión común de hábitats urbanos, rurales y naturales. Los resultados muestran que la urbanización aumenta los niveles de ácidos grasos y el daño oxidativo a los lípidos y afecta negativamente la condición corporal de las aves, lo que a su vez influye en la actividad de las enzimas antioxidantes. Entre otras posibilidades, esto podría deberse a la falta de disponibilidad de alimentos de buena calidad en los hábitats urbanos que evitan que las aves alcancen los requisitos fisiológicos durante el desarrollo, resultando en condición corporal, inmunocompetencia y capacidad antioxidante inferior. Además, los gorriones jóvenes infectados por *Plasmodium* tenían una menor proporción de ω-6 y más de ω-3 PUFAs, así como una menor actividad de las enzimas antioxidantes (GPx, SOD y GR). Estos resultados podrían reflejar que, debido a la baja actividad antioxidante en las aves infectadas, es probable que sobrevivan exclusivamente aquellas aves con niveles moderados de ácidos grasos proinflamatorios para lidiar contra la infección.

Además, los resultados de esta tesis muestran que las aves expuestas a dos fuentes diferentes de estrés, como son la urbanización y las infecciones por parásitos, pueden sufrir efectos deletéreos mayores. Las aves de hábitats naturales infectados por *Plasmodium* mostraron una relación ω-6/ω-3 más baja y tendieron a tener una proporción más alta de ω-3. Es probable que, ante una disponibilidad diferencial de ω-3 PUFA entre hábitats, solo los gorriones infectados de hábitats naturales promuevan una mayor respuesta inmunitaria antiinflamatoria hacia las infecciones por *Plasmodium* con por la consumición de alimentos ricos en ω-3 PUFA. Además, una mayor condición corporal de los gorriones juveniles se asoció con infecciones por *Plasmodium* y *Haemoproteus*, especialmente en hábitats urbanos. Estos resultados pueden explicarse por una tasa de mortalidad diferencial de aquellas aves con menor condición corporal que no pueden lidiar con la infección, consecuentemente, los individuos infectados que sobreviven a la infección estuvieran en una condición corporal más alta que los no infectados.

En resumen, en esta tesis aporto información novedosa sobre la dinámica de los parásitos transmitidos por vectores en ecosistemas antropizados y los efectos negativos conjuntos de la urbanización y las infecciones por parásitos en aves silvestres. Aquí destaco la importancia de incluir, en los estudios de impacto de la urbanización, hábitats con diferentes usos como los hábitats rurales, donde el efecto de las actividades humanas y animales domésticos sobre la vida silvestre puede diferir de las ciudades. Además, los niveles de contaminación, la disponibilidad de espacios verdes o la estabilidad de recursos puede diferir

ampliamente entre ciudades, lo cual puede determinar los efectos finales en la salud de la vida silvestre y en la interpretación que los investigadores pueden hacer a partir de ella del impacto de la urbanización. Este trabajo contribuye la creciente área de *Ecología Urbana* y ayuda a comprender mejor los efectos que tanto la urbanización como los patógenos tienen sobre la ecología y la evolución de la vida silvestre bajo el actual escenario de cambio global.

GENERAL SECTION

Introduction

In less than a century, humanity has almost triplicated its population reaching around 8 billion people in 2021, of which half of them live in urban areas. Human needs have led to a landscape transformation caused by the intensification of agriculture, animal husbandry and the expansion of cities (Bradley and Altizer, 2007; Grimm et al., 2000). These activities have changed the environmental conditions around the globe, affecting ecosystems and creating habitats with novel environmental characteristics (Meyrier et al., 2017; Seress and Liker, 2015). Habitat anthropization has been linked with a reduction of biodiversity and the alteration of the interactions between organisms, including the transmission of diseases (Bradley and Altizer, 2007; Croci et al., 2008; Seress and Liker, 2015; Sol et al., 2014). Due to the inability to exploit resources and thrive with the stressful and risky environment of anthropic habitats, only a reduced number of invertebrates, mammals, birds, reptiles and amphibians are able to survive, but once they stablish, they can even reach high abundances than in natural habitats (Callaghan et al., 2021; Kurucz et al., 2021; Mckinney, 2002; Meyrier et al., 2017; Seress and Liker, 2015; Sol et al., 2014). Nevertheless, urban habitats could represent ecological traps for wildlife due to the mismatches between animal perception of attractive urban features, and the negative impacts and costs these impose on fitness (Isaksson, 2020; Meyrier et al., 2017; Pollock et al., 2017; Sumasgutner et al., 2014). During the nineteens, urban ecology emerged as a novel research area due to the urgent need to understand how species respond and adapt to urban environments and to characterize the mechanism behind urban-dwelling animals (Chace and Walsh, 2006; Grimm et al., 2000; MacGregor-Fors et al., 2020; Ouyang et al., 2018; Sepp et al., 2018). However, those studies have usually focussed on the effects of urbanization on individual fitness or on disease transmission, but there is a lack of studies that consider both factors at the same time. Thus, this thesis is framed in the area of urban ecology aiming to analyse the impact of urbanization on vector-borne pathogens transmission dynamics and test the potential synergic effect of both urbanization and parasite infection on wild birds health.

Effects of urbanization on birds

Cities provide favourable conditions for birds such as milder climate (Saaroni et al., 2000), high availability of nesting sites (Sumasgutner et al., 2014) and reduced density of predators (Møller and Díaz, 2017; but see Dulisz et al., 2016). Substantial differences in the composition of urban and rural avian communities have been recorded across the world (Croci et al., 2008; Leveau, 2021; Mbiba et al., 2021). Urban dwelling species may benefit from access to highly predictable and stable resources through the year like food and water (Fokidis et al., 2008; Pithon et al., 2021; Shochat et al., 2006). Meanwhile, in less modified habitats or in natural ones, resource abundance or availability will depend, to a larger extent, on environmental factors like hydrological and vegetation characteristics or climatic conditions (Andersson et al., 2018; Chamberlain et al., 2009; Hanson et al., 2020). Overall, resource availability in urban areas can improve the health and increase the survival of individuals of urban dwelling species (Chamberlain et al., 2009; Dulisz et al., 2016) or at least, buffer the negative impact of urbanization (Sumasgutner et al., 2014). Consequently, there is an intense debate on whether urban habitats represent ecological traps or safe spaces for species, and what characteristics allow urban-dwelling species to thrive in urban areas (Zuñiga-Palacios et al., 2021). The progress in this area should deal with the wide geographic spreading of studies around the globe, harbouring areas with different environments. However, the very heterogeneous approaches used across studies to measure urbanization makes it difficult and leads to highly contrasting results between studies (Box 1).

Box 1. Could be urbanization equally measured around the globe?

The studies of urban ecology consulted during the elaboration of this thesis include research done along America, Europe, Africa, or Asia, in cities placed in a wide range of bioclimates and with very different urbanization intensities and structure. The term *urban* includes localities from densely populated areas that relies in vertical development, to less populated areas based on single family houses with wide gardens and vegetation. In these works, urbanization is measured either qualitatively or quantitatively. Usually, localities are classified regarding visual characteristics and comparatively with the other localities considered in the study. *Urban* or *city* is used when referring to the most urbanized area, and *natural* or *forest* when referring to the less anthropized areas in the study. Intermediate localities are classified as *rural, suburban, peri-urban, plantation, ranch* or even as *non-urban,* to refer to the places that are not previously classified as *urban* or *cities* and harbouring a variety of land uses. Alternatively, authors quantify urbanization in a continuous scale. For example, satellite information tools are widely extended to measure the percentage of area covered by specific land uses in a defined parcel (i.e., building, roads, agriculture, vegetation, forest, water, dry land…), which data can be used to calculate a unique value of urbanization. In some cases, abiotic factors such as level of noise, light, and pollutants or biotic factors like human population density, are also considered. The variation of environmental and climatic traits as well as the different urban structure used along the world, makes very difficult to stablish whether a specific characteristic of a city is positive, negative, or indifferent for urban dwelling individuals. For example, the presence of artificial water can be a very important factor in cities surrounded in arid environments or deserts but be indifferent in places with high precipitation and water availability in the surrounding natural areas. Furthermore, a proper replication of habitats is necessary within studies. The comparison of a single natural and a single urban area, makes impossible to determine if the differences found are due to urbanization or are just reflecting other distinction between localities unrelated to it. The use of such different approaches to evaluate urbanization and the lack of homogenization, makes difficult to compare information. These inconvenient may explain the contrasting results found among studies dealing with the measurement of the impact of urbanization to wildlife.

Urbanization may impact organisms by affecting both the availability and quality of the food supply (Costantini, 2010; Delhaye et al., 2018a; Romieu et al., 2008), and by the presence of pollutants and other environmental stressors derived from human activities (Bichet et al., 2013; Herrera-Dueñas et al., 2014; Koivula et al., 2011; Romieu et al., 2008; Salmón et al., 2018). Food with an anthropogenic origin (i.e., human waste), is usually rich in carbohydrates and fats but poor in protein content and if consumed regularly, can have negative consequences on the fitness of wild birds (Heiss et al., 2009; Mckinney, 2002; Meyrier et al., 2017). Invertebrates rich in proteins usually form an important fraction of the diet of passerine chicks, but the lack of suitable prey in anthropized environments lead to the substitution by human food (Anderson, 2007; Finke et al., 2020; Pollock et al., 2017). This change strongly influences nesting success, fledgling growth, and survival, and thereby, population demography (Anderson, 2007; Peach et al., 2015, 2014; Seress et al., 2012). Nutrient rich food is especially important during early life stages to fulfil physiological requirements during development, to grow faster, and to have a higher body condition and immunocompetence (Chamberlain et al., 2009; Peach et al., 2018; Seress et al., 2020; Twining et al., 2016b, 2018).

Additionally, birds living in urban habitats are exposed to toxic chemicals, heavy metal particles, and other pollutants such as NO_x, SO_2 or O_3, derived from burning fossil fuels, that have been demonstrated to had toxic and deleterious impacts in the organisms (Herrera-Dueñas et al., 2017; Isaksson, 2010; Koivula et al., 2011; Salmón et al., 2018). Reactive oxygen species (ROS) are a group of chemical substances that may cause oxidative damage to biomolecules such as proteins, lipids, or DNA (Li et al., 2016). In order to avoid pathological conditions, organisms present antioxidant systems that include both enzymatic and non-enzymatic compounds (i.e., vitamins, obtained through diet) that prevent, reduce, and repair ROS-induced damage (Costantini and Verhulst, 2009; Li et al., 2016; Sorci and Faivre, 2009). ROS are either derived from an internal source, as by-products of basal metabolism and inflammatory responses to fight external aggressions (Costantini, 2019; Isaksson, 2015; Sorci and Faivre, 2009), or, from a synthetic external source, like anthropic activities (Herrera-Dueñas et al., 2017; Salmón et al., 2018). Thus, when the balance between antioxidants molecules and pro-oxidant like ROS is lost, the oxidative stress increase and could finally develop into oxidative damage to biomolecules (Costantini and Verhulst, 2009; Li et al., 2016). Indeed, birds living in urban areas suffer an increase of oxidative stress due to higher exposure to environmental stressors (Herrera-Dueñas et al., 2014).

The detrimental effects of habitat anthropization on life history-traits of birds have been reported in different species, including those apparently thriving in urban areas (Carvajal-Castro et al., 2019; Croci et al., 2008; Ibáñez-álamo et al., 2017; Sol et al., 2014). Urbanization negatively affects the body size and condition of birds (Jiménez-Peñuela et al., 2019; Liker et al., 2008; Meillère et al., 2015), their reproductive success and survival (Chamberlain et al., 2009; Sepp et al., 2018; Sumasgutner et al., 2014) and even alter critical processes such as feather moult or development of sexual ornaments (Hutton et al., 2021; Tomášek et al., 2016). Several studies have identified the impact of urbanization on different life stages of birds using the quantification of a large variety of biomarkers. For example, telomers are non-coding DNA that maintain genome stability and determines cells senescence (O'Sullivan and Karlseder, 2010). During early life stages, the shortening of telomere length was larger in great tit nestlings from urban than rural habitats (Salmón et al., 2016). Additionally, individuals with shorter telomere length were more prone to disappear in urban habitats compared to rural ones (Salmón et al., 2017). DNA methylation regulates the expression of associated genes and genome stability and is a useful marker in the evaluation of epigenetic changes (Bird, 2002). Watson et al. (2021) recently found that there was an ubiquitous hypermethylation of the DNA of great tits living in urban habitats, compared to conspecifics living in nearby forest habitats, which had a lower DNA methylation. This epigenetic divergence was pointed out to reflect environmental differences in diet and ROS exposure among populations, both considered as key drivers of the increasing differentiation of urban and non-urban birds (Watson et al., 2021). Fatty acids are obtained through food and play an important role in biological structures and in processes such as oxidative stress and immune responses (Arnold et al., 2015; Hulbert et al., 2005; Isaksson et al., 2017). Differences in fatty acids composition can be observed between birds from urban and rural areas in different species of sparrows and tits, initially in eggs and after, during adult life (Andersson et al., 2018, 2015; Isaksson et al., 2017; Toledo et al., 2016). In addition, great tits from urban habitats presented higher level of expression of genes related to immune, inflammatory and antioxidant responses, than those breeding in natural habitats (Watson et al., 2017). Differences in body size, body condition and feather quality have been also found between house sparrows from urban and rural habitats (Dulisz et al., 2016; Meillère et al., 2017). Thus, strong evidence from different urban-dwelling passerine birds supports that urbanization act as an important evolutionary selective force. This has led to behavioural, physical and physiological changes in urban individuals, resulting even in the rapid evolution of genetic differences in some species regarding their non-urban conspecifics (Markowski et al., 2021; Ouyang et al., 2018; Salmón et al., 2021).

Parasite infections across urbanization gradients

The simplification of habitat structure caused by urbanization did not only affect the life history-traits, abundance and composition of the communities of vertebrates (Biard et al., 2017; Chace and Walsh, 2006; Crooks et al., 2004; Evans et al., 2009; Liker et al., 2008) but also, of the hematophagous insects living in these areas and, consequently, of the vector-borne pathogens they transmit (Ciadamidaro et al., 2016; Ferraguti et al., 2016a; Santiago-Alarcon et al., 2013). Urbanization can alter the incidence and distribution of parasites, but also the susceptibility of vertebrates to its infectious diseases affecting host immunocompetence (Bradley and Altizer, 2007; Calegaro-Marques and Amato, 2014; Ferraguti et al., 2018; Ishtiaq, 2021; Jiménez-Peñuela et al., 2021; Muriel et al., 2021; Patz et al., 2008).

Haemosporidian represent a major selective force potentially regulating host populations (Newton, 1998; Rivero and Gandon, 2018). Different studies have reported clear deleterious effects of blood parasite infections on different life-history traits of birds such as health (Marzal et al., 2008; Merino et al., 2000), longevity (Martínez-de la Puente et al., 2010), reproductive success (Cuevas et al., 2021; Marzal et al., 2005; Merino et al., 2000), hormone modulation (Names et al., 2021) and oxidative stress (Arriero and Møller, 2008; Martínez-de la Puente et al., 2011; Morales et al., 2006). Indeed, correlative and experimental studies indicate that malaria related parasites are common sources of physiological stress (del Cerro et al., 2010; Martínez-de la Puente et al., 2011; Merino et al., 1998; Videvall et al., 2015). During the course of infection, organisms develop inflammatory responses that produce cytotoxic compounds that act like ROS, increase the oxidative stress and thus, harm the host unless the antioxidant system activates (Costantini and Verhulst, 2009; Sorci et al., 2013; Sorci and Faivre, 2009). Even this can be costly, individuals unavailable to produce inflammatory responses are more susceptible to suffer increased infection-induced mortality (Sorci and Faivre, 2009). Additionally, immune responses to fight off infections are energetically costly, with only those individuals with higher body condition being able to develop more effective responses (Navarro et al., 2003). This trade-off aggravates in cities, where the activation of constitutive immunity is even more costly, confirmed by a decrease in haptoglobin production and a loss of body mass in urban great tits infected by haemosporidians (Bailly et al., 2016). A poor diet, both in quality or quantity, such as those consumed by different avian species living in urban habitats, may increase host's susceptibility to infectious diseases (Cornet et al., 2014). Intrinsic and extrinsic factors largely determine host susceptibility to infection, defining its ability to modulate immune responses to fight off infections and the potential costs of these responses

(Arriero et al., 2008; Delhaye et al., 2018a; Morales et al., 2006). It is difficult to isolate the effects of infections from those of food-shortages or other environmental conditions that may favour disease transmission or modify the trade-offs of birds, finally affecting their tolerance and resistance to infections (Arriero et al., 2018; Newton, 1998). However, understanding the ecology of pathogens in anthropized areas and the consequences of both, anthropization and infection, on the health of avian hosts, is essential to manage the risk of pathogen transmission in altered environments, especially under the current global change scenario that may alter the distribution of hosts, vectors and pathogens (Bradley and Altizer, 2007; Cornet et al., 2014; Isaksson, 2020).

Study models

The bird study model: House sparrow

House sparrows (*Passer domesticus*) are small passerines ranging between 14-16 cm length, with a body mass range from 24 to 39.5 g and with different sex plumage coloration between adult male and female (Fig. 1) (Svensson, 2009). This species is sedentary, abundant and has a widespread and ubiquitous distribution around the globe (Anderson, 2007; Hanson et al., 2020; Summers-Smith, 1963, 1956). House sparrows have a large native distribution area across Europe, Africa, Asia and an introduced distribution area in Oceania, North and South America and South Africa (Anderson, 2007; De Laet and Summers-Smith, 2007; Hanson et al., 2020). This species usually breeds during spring and summer in their native range (Anderson, 2007), but with temporal differences between areas. After hatching, adults feed chicks with an insect-based diet and afterwards, they also introduce grain (Anderson, 2007). Sparrows can be found in a variety of climatic areas and habitats, although is uncommon in forest habitats, scrublands or wetlands, as it usually occurs within or nearby anthropogenic habitats and human settlements (Bichet et al., 2015; SÆtre et al., 2012; Seress and Liker, 2015). Moreover, this species has a low dispersal rate with a restricted home range radius of 1000-2000 m with only around 10% of the juveniles individuals dispersing over 2000 m from their natal locality (Snow and Perrins, 1998; Summers-Smith, 1963, 1956).

Figure 1. House sparrows of different age classes and sexes captured in the context of the studies of this thesis. Adult female (left), adult male (middle) and juvenile (right).

We selected this species due to its historical association with humans, for which is considered one of the best examples of urban exploiter and known to thrive taking advantage of food resources and cavities for nesting both, in and, close to human settlements (Hanson et al., 2020; Mckinney, 2002; Meillère et al., 2015). However, since the 1970s, house sparrow populations are undergoing a dramatic decline across Europe, especially in cities (Anderson, 2007; De Laet and Summers-Smith, 2007; Hanson et al., 2020), including Spain (Murgui and MacIas, 2010). Among other factors, nutritional constrains, intensive animal husbandry and agriculture, pollution, pesticides and infectious diseases caused by blood parasites, have been reported as potential causes for this population decline (Bichet et al., 2013; Dadam et al., 2019; Herrera-Dueñas et al., 2017, 2014; Meillère et al., 2017; Meyrier et al., 2017; Peach et al., 2018; von Post et al., 2012).

Blood parasites

Avian malaria parasites of the genus *Plasmodium* and the related genera *Haemoproteus* and *Leucocytozoon*, are widespread vector-borne parasites commonly found infecting birds (Atkinson et al., 1991; Valkiūnas, 2005) and frequently used as models in the study of the ecology of vector-borne pathogens (Fecchio et al., 2020; Rivero and Gandon, 2018). These parasites are transmitted by different insect vectors with distinct environmental requirements (Atkinson et al., 1991; Valkiūnas, 2005). *Plasmodium* parasites are transmitted by mosquitoes (*Culicidae*) with most species thriving in small temporary water bodies (Becker et al., 2020, 2010). Among other species, *Culex pipiens* is a competent vector highly relevant for the transmission of avian *Plasmodium* in southern Spain (Ferraguti et al., 2020; Gutiérrez-López et al., 2020; Roiz et al., 2014). Biting midges (*Culicoides*) are the main vectors of *Haemoproteus* (*Parahaemoproteus*) parasites (Purse et al., 2015), which require wet soils for breeding. Previous studies in Spain suggest that different *Culicoides* species may be involved in the transmission of *Haemoproteus*, with *Culicoides circumscriptus* and *Culicoides paolae* likely acting as important vectors in Andalusia (Ferraguti et al., 2013; Martínez-de la Puente et al., 2009b; Veiga et al., 2018). Finally, blackflies (*Simuliidae*), the main vectors of *Leucocytozoon* parasites, require constant running water bodies for breeding (Ciadamidaro et al., 2016). Little is known about these vectors in Andalusia, but recent evidence supports the potential role of *Simulium rubzovianum* as a vector of *Leucocytozoon* parasites in the south of Spain, especially in birds breeding in nest boxes (Chakarov et al., 2021).

House sparrows are frequently infected by haemosporidian parasites (Bichet et al., 2013; Ferraguti et al., 2018), with a highly variable prevalence of infection in different populations across its distribution area (Marzal et al., 2011). Up to now, 39 genetically different lineages of *Plasmodium*, 13 lineages of *Haemoproteus*, and 8 lineages of *Leucocytozoon* have been recorded infecting house sparrows according to MalAvi database (accessed July 18th) (Bensch et al., 2009). These characteristics make the house sparrow suitable study model to assess the joint impact of urbanization and parasite infections on individual traits (Liker et al., 2008). Especially, juveniles individuals, reflects the environmental conditions occurring during spring and summer of the sampling year and the parasites circulating in the area during this period, as they may be infected during nestling and post-fledgling stages (Cosgrove et al., 2008; Fecchio et al., 2020; Valkiūnas, 2005).

Objectives

The main goal of this thesis was to determine how the dynamics of blood parasites are affected by the habitat anthropization and how the joint effects of this anthropization and parasite infections, impact on the health of wild birds. To do that, I addressed two main questions that frame into two different sections (Fig. 2). **Section 1:** Does habitat anthropization affect the transmission of avian blood parasites? **Section 2:** Does habitat anthropization and blood parasite infections affect birds health?

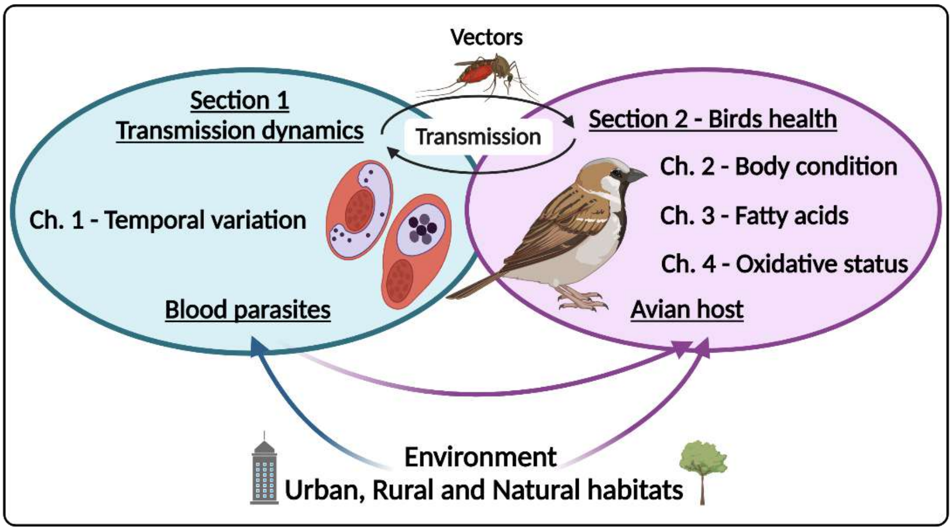


Figure 2. Schematic representation of the approach used in this thesis to test the relationships between anthropization, the transmission dynamics of blood parasites and the joint effects of anthropization and parasite infection on different measures of wild birds health. In the **Section 1**, I compared the temporal variation of blood parasite infections in wild birds from habitats with different anthropization levels (**Ch. 1**). In the **Section 2**, I identified the impact of parasite infections and landscape anthropization along urban, rural and natural habitats on three major variables reflecting the health of birds, namely body condition (**Ch. 2**), the composition of plasma fatty acids (**Ch. 3**) and the oxidative status (**Ch. 4**) estimated from birds blood samples. 'Ch.' followed by numbers indicates the chapter of the thesis in which these relationships are studied. Created with BioRender.com.

In **Section 1 (Chapter 1)**, I studied the transmission dynamics of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* parasites along two consecutive years (2013 and 2014). Firstly, I evaluated how parasite prevalence and lineage richness, two ecological parameters reflecting parasite infections dynamics in birds, varied among and between these two years in urban, rural and natural environments. Avian malaria prevalence and lineage richness usually fluctuate between years, especially linked to climatic and environmental variation (Lachish et al., 2011; Wood et al., 2007) and contrasting results have been found when comparing birds populations from cities and less anthropized habitats (Delgado-V. and French, 2012; Santiago-Alarcon et al., 2019; Sehgal, 2015). However, the temporal stability of parasite communities in altered environments remains an open question that has been addressed in this chapter.

Secondly, in **Section 2**, I analysed the impact of both habitat anthropization and blood parasite infections on different variables reflecting the health of wild house sparrows. I evaluated the body condition (**Ch. 2**), the composition of fatty acids (**Ch. 3)** and the oxidative status (**Ch. 4**) of birds uninfected and infected by avian malaria parasites, across an urbanization gradient, including populations from urban, rural, and natural habitats. Hence, in **Chapter 2**, I compared the body condition of individuals in relation to their infection status by *Plasmodium*, *Haemoproteus* and *Leucocytozoon* parasites, across an urbanization gradient. Different methods have been proposed to control for, or remove, the effects of individual size on body mass (i.e., Bókony et al., 2012; Peig and Green, 2009). Although the use of bird body mass as a measure of body condition has been debated, this variable is easy to measure, repeatable and allows to describe energy and nutrient reserves (Labocha and Hayes, 2012). Here, the individual size (wing length) was included as a covariate on the analyses with body mass as dependent variable (Garcia-Berthou, 2001). Indeed, when body condition was used as independent variable in other chapters, we use a body condition index estimated following Peig and Green, (2009) (**Ch. 1** and **3**) or only body mass when data of bird size was not available due to plumage moult (**Ch. 4**).

Thirdly, in **Chapter 3**, I studied the serum fatty acids (FA) composition of house sparrows according to their infection status by *Plasmodium* and from populations across an urbanization gradient. FA are physiological components that plays an important role as biological structures, like phospholipids in cell membranes, and in processes such as oxidative stress, inflammatory and immune responses (Arnold et al., 2015; Hulbert et al., 2005; Isaksson et al., 2017). The food type consumed by birds would influence the intake of different FA while physiological processes, such as metabolism, selective transportation and use (Alagawany et al., 2019; Hulbert et al., 2005; Hulbert and Abbott, 2012; Price and Valencak, 2013) would influence FA composition in the organism, especially in the circulatory system (Finke et al., 2020). FA usually maintain their original structure, which make them useful as dietary tracers (Twining et al., 2016) and provides support for investigating bird's physiological condition alongside with nutritional status to understand population tolerance towards parasitic infections in landscapes of varying degree of urbanization.

Finally, in **Chapter 4**, the oxidative status of juvenile house sparrows was measured across an urbanization gradient including individuals uninfected and infected by *Plasmodium*, *Haemoproteus* and *Leucocytozoon*. During the course of infection, organisms develop inflammatory responses during which cells of the immune system produce cytotoxic compounds that increase ROS (Sorci et al., 2013; Sorci and Faivre, 2009). Additionally, pollutants or other environmental stressors derived from human activities, such as burning fossil fuels, are toxic and deleterious substances that can also increase ROS in birds from urban habitats (Bichet et al., 2013; Herrera-Dueñas et al., 2017, 2014; Isaksson, 2010; Koivula et al., 2011; Romieu et al., 2008; Salmón et al., 2018). Thus, our aim was to identify the impact of both habitat, parasite infections and their interaction, on the physiological oxidative status of wild birds. For this, we evaluate both the oxidative damage caused to lipids and the antioxidant capacity of birds, estimated through the concentration of three different antioxidant enzymes involved in oxidative stress control. Overall, these three chapters contribute to the understanding of the impact of landscape anthropization and parasite infections on the health of birds.

Material and methods

Study area and bird sampling

The study was conducted in Andalusia, in south-west Spain. This area is characterized by a Mediterranean climate with mild-warm temperatures (16.8ºC mean annual temperature). The area is characterized by long, dry, and hot summers followed by short winters with low precipitation (605 mm annual mean), which high variability between seasons and years, makes water availability a main limiting factor.

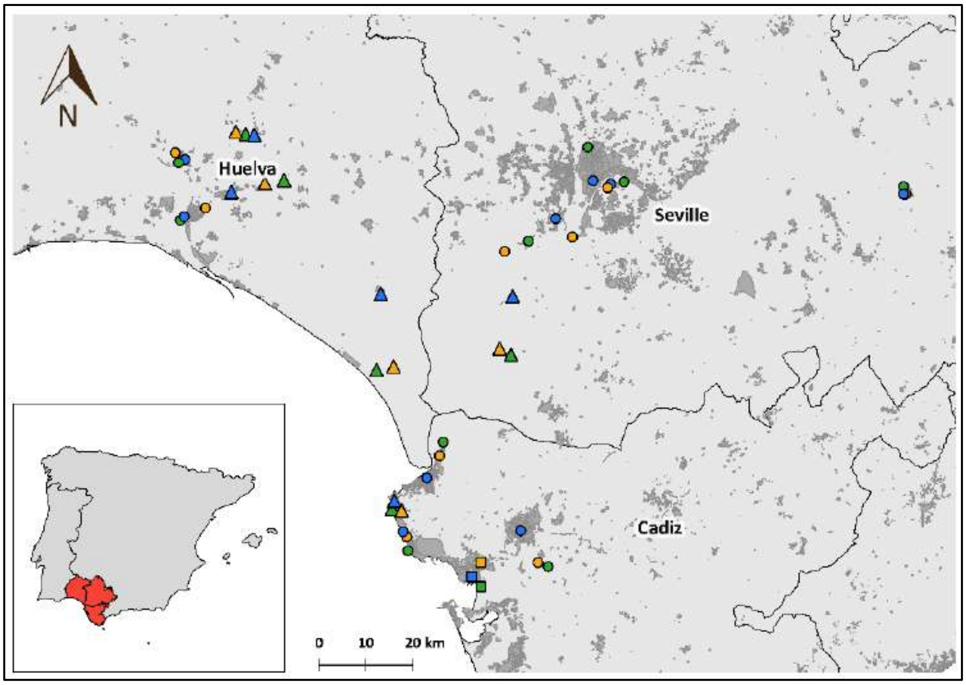


Figure 3. Map of the study area in south-west Spain. Sampled localities included urban (blue), rural (yellow), and natural (green) habitats. Urbanized areas are highlighted in dark grey in the map. **Chapter 1** includes all localities of the Huelva province sampled during two consecutive years (2013 and 2014). **Chapter 2** includes samples from all the 45 localities (all figures). **Chapter 3** includes samples of birds from localities represented with triangles. **Chapter 4** includes all localities except for those represented with squares.

Birds from 45 localities were sampled in the provinces of Seville (N=15) and Cadiz (N=15) during 2013 and in Huelva (N=15) during both 2013 and 2014 (Fig. 3). Geographically close localities were grouped in triplets harbouring one urban, one rural, and one natural habitat (see Landscape characterization section for further information). The average distance within localities of the same triplet was 5739.87 m (±SD 4465.12 m). The geographical structure of the triplets was maintained in all chapters. However, the number of sampling localities and years included varied between the chapters of this thesis. In **Chapter 1**, the 15 localities from Huelva province sampled during two consecutive years were included. **Chapter 2** includes information from all the 45 localities sampled during 2013. In **Chapter 3**, due to the limited funding available to run the eco-physiological analyses, there was a reduction of the number of localities included, with only 5 triplets being analysed. We included nine localities from Huelva (3 triplets), three localities from Seville (1 triplet) and three localities from Cadiz (1 triplet). In **Chapter 4**, a total of 42 localities (14 triplets) sampled in 2013 were included. One triplet had to be excluded from the oxidative stress analyses because of the low number of juvenile male house sparrows captured in one locality of the triplet, consequently, the complete triplet was excluded from the analyses.

Wild house sparrows were captured from July to October in 2013 and from June to August in 2014 by a maximum of three teams of specialized ringers (Fig. 4). Birds were captured using mist-nests and a bird call playback recorder. Birds were ringed with a numbered aluminium ring, and, when possible, visually sexed and aged according to their plumage characteristics and skull ossification (Svensson, 2009). Birds were classified as yearlings (birds younger than one year, Euring code 3), adults (birds older than one year, Euring code 4), and as unknown age (Euring code 2). Birds of the last category were excluded from the analyses. Birds were weighted with an electronic balance (to the nearest 0.1 g) and their wing length was measured with a metal ruler (to the nearest 1 mm). A blood sample was obtained from the jugular vein of each bird using sterile syringes, never exceeding 1% of their body mass. Blood samples were transferred to Eppendorf tubes and preserved in cold-boxes during the field work. Subsequently, samples were transferred to refrigerators and kept at 4ºC until centrifugation for 10 min at 1700g (4000 rpm) the next morning. The serum and cellular fractions were separated and frozen at -80ºC and -20ºC, respectively, until further analyses.

Figure 4. Process of extraction from the mist-net, ringing, wing length measure, body mass measure and blood extraction of house sparrows (from up left to right down).

Landscape characterization

The categorization of the localities was based on visual inspection of the habitat, being classified as urban, rural or natural habitats. Urban habitats were characterized by higher density of human populations than rural and natural habitats from the same triplet. Rural habitats were defined by a higher density of livestock than urban and natural ones from the same triplet. Natural habitats corresponded to better conserved areas where wildlife predominated over both humans and livestock. This characterization was used in all chapters of this thesis.

Additionally, complementary data of landscape use was estimated and used in **chapter 2**. First, an urbanization score was obtained for each locality with a semi-automated software (http://keplab.mik.uni-pannon.hu/en/ urbanization-index) following the procedure described in Czúni et al. (2012), Lipovits et al. (2015), and Seress et al. (2014). Briefly, satellite images were obtained in '.jpeg' format from Google Maps covering an area of 1 square km around each bird sampling point. After marking some reference points, the software estimates the proportion of buildings, vegetation, and paved road coverture, which are then combined in a Principal Components Analysis (PCA) to obtain an 'urbanization score'. This ranges from low and negative values regarding less urbanized areas, to positive and higher values for more intensely urbanized ones. Moreover, the percentage of the total area occupied by agricultural, natural and urban lands was measured using the cartography from the study area (http://www.juntadeandalucia.es/institutodeestadisticaycartografia/DERA/) considering a 2000 m radius buffer around the bird sampling site using the software ArcGis v10.2.1 (ESRI, Redlnad). Also, human population density was estimated as the number of people living in a grid of 250m^2 according to the data registered on 1st January 2013 by the Institute of Statistics and Cartography of Andalusia.

Laboratory analyses

Genomic DNA was extracted from the blood cellular fraction using the Maxwell®16 LEV System Research (Promega, Madison, WI, USA). For those birds not sexed morphologically in the field, sex was determined following the protocols by Griffiths et al. (1998, 1996). The infection status of birds and blood parasite identity were determined through the amplification of a 478 bp fragment of the haemosporidian mitochondria cytochrome *b* gene (Hellgren et al., 2004).

Negative samples were analysed twice to avoid false negatives (McClintock et al., 2010). Both negative controls for the PCR reactions (at least one per plate) and DNA extractions (one per 15 samples) were included in the analysis. PCR amplifications were resolved in 1.2% agarose gels and positive amplifications were sequenced. The parasites were identified by comparison with sequences deposited in public databases including GenBank (National Center for Biotechnology Information) and MalAvi (Bensch et al., 2009). This information was included in the four chapters of this thesis.

Fatty acids (FA) analysed in **Chapter 3** were extracted from serum samples following the protocol described in Andersson et al. (2015) and measured by Gas Chromatography/Mass Spectrometry (GC/MS). The relative proportion of the three main FA groups namely saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), the PUFA groups Omega 3 and 6 (ω-3 and ω-6), and the absolute FA concentration (FA_{tot}) were estimated. In addition, we also included in our analyses two biomarkers, namely the ω-6/ω-3 ratio and the Peroxidation Index (PI), a proxy for measure the susceptibility of lipids to peroxidation (i.e., susceptibility to oxidative damage).

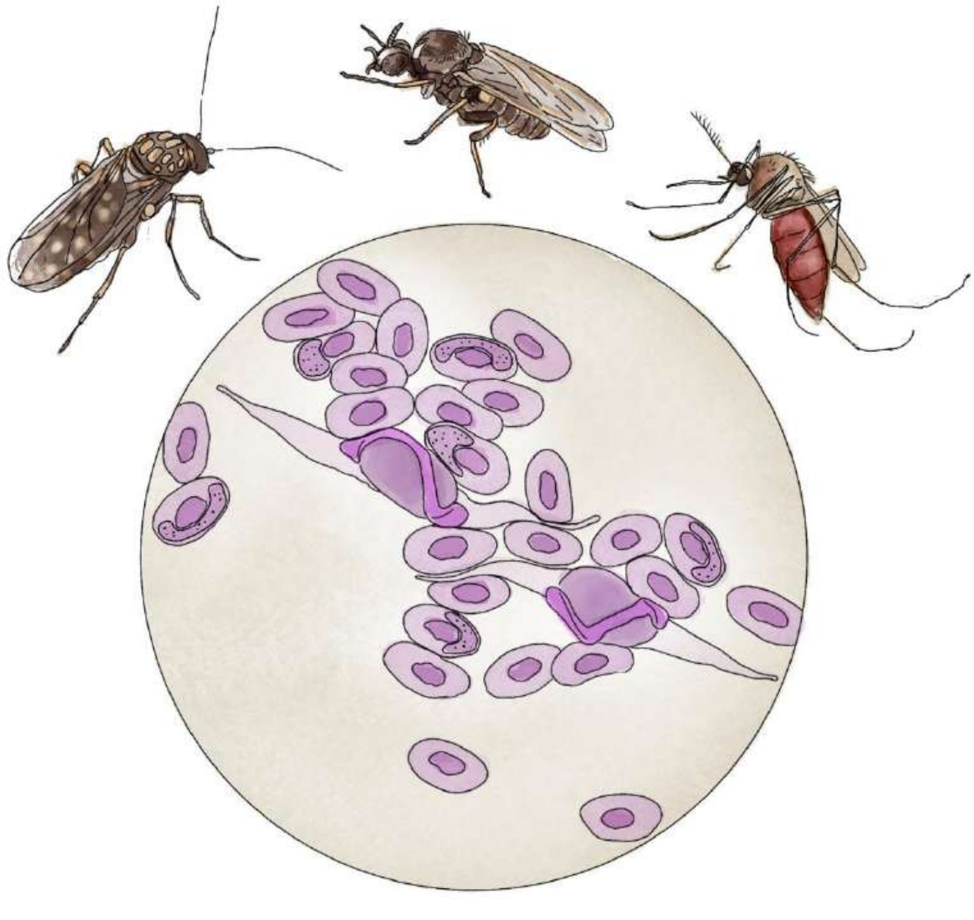
Finally, in **Chapter 4**, four different markers were studied from the blood cellular fraction of birds to evaluate their oxidative status. These markers included the thiobarbituric reactive substances (TBARS), that measures the oxidative damage to lipids and the activity of the superoxide dismutase (SOD), the glutathione peroxidase (GPx) and the glutathione reductase (GR), enzymes implied in the redox system and the antioxidant capacity of birds.

SECTION 1

TRANSMISSION DYNAMICS

CHAPTER 1

URBANIZATION EFFECTS ON TEMPORAL VARIATIONS OF AVIAN HAEMOSPORIDIAN INFECTIONS



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Environmental Research (2021), 111234

Graphical abstract

Highlights:

- *Plasmodium, Haemoproteus* and *Leucocytozoon* infection dynamics respond differently through habitat types and time.
- The prevalence of *Haemoproteus* and *Leucocytozoon* were higher in 2013 than in 2014 and positively correlated between years.
- Lineage richness of rural areas differed yearly for *Haemoproteus* and was negatively correlated within years for *Leucocytozoon.*
- The *Plasmodium* prevalence of infection and lineage richness were similar between years and habitat types.
- Parasite lineages were homogeneously distributed and had similar prevalence across habitat types and sampling years.

Keywords: *Haemoproteus*, Landscape change, *Leucocytozoon*, *Passer domesticus*, *Plasmodium*, Vector-borne diseases.

Abstract

Urbanization is an important human-driven process that leads to biodiversity loss and alters the interactions between organisms, including disease transmission. Although urbanization affects both host and vector communities, the effects on vector-borne pathogens are still poorly understood. Here, we monitored variation in prevalence and richness of three common blood parasites in birds (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) from localities with different land uses (urban, rural and natural) during two consecutive years (2013 and 2014). Overall, 1400 juvenile house sparrows from 15 localities in southern Spain were included in this study. *Haemoproteus* and *Leucocytozoon* prevalence was higher in 2013 than in 2014, particularly in urban and natural habitats for the case of *Leucocytozoon*. Prevalence was correlated between years for *Haemoproteus* and *Leucocytozoon*, independently of the habitat. Additionally, rural habitats harboured significantly higher *Haemoproteus* lineage richness compared to urban and natural habitats during 2014. *Leucocytozoon* lineage richness was negatively correlated between years in rural habitats but positively correlated in urban and natural habitats in comparison. Parasite lineages found in birds were homogeneously distributed along habitats and years and the common lineages prevalence were not influenced by them. Our results highlight different patterns of infection depending on the parasite genera probably related to the composition and density of vector communities. The specific reproductive environmental requirements of the different groups of vectors involved in the transmission may be affected by climatic conditions and landscape features.

Introduction

The expansion of urban areas has important environmental impacts that affects organism survival, leads to biodiversity loss and alters the interactions between organisms, including disease transmission (Bradley and Altizer, 2007; Seress and Liker, 2015; Sol et al., 2014). The simplification of habitat structure caused by urbanization affects the abundance of hematophagous insects and the susceptibility of hosts to infectious diseases, consequently altering the spread of vector-borne parasites (Calegaro-Marques and Amato, 2014; Patz et al., 2008). Changes in abiotic and biotic factors driven by humans have been proposed to affect life history-traits, abundance and composition of the communities of both vectors (Ciadamidaro et al., 2016; Ferraguti et al., 2016; Santiago-Alarcon et al., 2013) and vertebrate hosts (Biard et al., 2017; Chace and Walsh, 2006; Crooks et al., 2004; Evans et al., 2009b; Liker et al., 2008). Nevertheless, human activities also provide alternative resources that can be exploited by anthropophilic species (Møller and Díaz, 2017). Such is the case of the house sparrow, a passerine bird that for centuries has lived together with humans taking advantage of anthropogenic food resources and cavities for nesting (Anderson, 2007; Laet and Summers-Smith, 2007). This may be also the case of invertebrates such as *Culex pipiens* and *Aedes albopictus*, hematophagous mosquitoes that have found in urbanized areas suitable habitats for breeding (Ferraguti et al., 2016; Gangoso et al., 2020; Li et al., 2014; Wilke et al., 2019). Urban resources are stable throughout time and are highly predictable for urban dwelling species, while in less modified habitats or in natural ones, the presence or availability of these resources will depend, to a larger extent, on environmental factors like climate (Becker et al., 2010; Chamberlain et al., 2009; Roiz et al., 2014). Thus, the composition and stability of vector and host communities in urbanized landscape may influence the transmission of vector-borne parasites through space and time.

Avian malaria parasites of the genus *Plasmodium* and the related genera *Haemoproteus* and *Leucocytozoon*, are widespread haemosporidians commonly found infecting birds (Valkiūnas, 2005) and frequently used as models in the study of the ecology of vector-borne pathogens (Fecchio et al., 2020; Rivero and Gandon, 2018). Different authors have compared the prevalence, richness and diversity of these blood parasites in bird populations from cities and natural habitats (Delgado-V. and French, 2012; Santiago-Alarcon et al., 2019; Sehgal, 2015), often obtaining contrasting results. While some authors found a lower prevalence of infection in birds from urban than from non-urban habitats (Evans et al., 2009a; Geue and Partecke, 2008; Santiago-Alarcon et al., 2020), other studies reported the opposite pattern (Belo et al., 2011; Bradley and Altizer, 2007; Hernández-Lara et al., 2017; Tinajero et al., 2019), or even no significant differences (Bichet et al., 2014; Ferraguti et al., 2018; Jiménez-Peñuela et al., 2019). Moreover, the richness of blood parasite lineages circulating in an area is associated to the richness of avian species present in a locality (Ferraguti et al., 2018). Consequently, given the reduced number of avian species present in urban habitats (Clark et al., 2018; Shochat et al., 2006), urbanization may not only affect prevalence of parasites but also result in a lower richness of blood parasites (Hernández-Lara et al., 2020; but see Fariello, 2019).

Studies in wild bird populations have shown that avian malaria prevalence and lineage richness usually fluctuate between years linked to climatic and environmental variation that may affect both vector and host populations (Lachish et al., 2011a; Wood et al., 2007). However, the temporal stability of parasite communities in altered environments remains an open question. Here, we analysed the relationship between landscape use and the temporal variation in two parameters reflecting parasite infections dynamics in birds of bird infections, namely parasite prevalence and lineage richness. To do that, we used the house sparrow (*Passer domesticus*) as study model species, based on its ubiquity and low dispersal rate (Anderson, 2007; Hanson et al., 2020; Summers-Smith, 1956). Specifically, we focused on juvenile birds as they may be infected during nestling and post-fledging periods, thus reflecting the parasite circulation in the area during the year of sampling (Cosgrove et al., 2008; Fecchio et al., 2020; Valkiūnas, 2005). We sampled birds from 15 localities with different landscape uses during two consecutive years. We tested whether the prevalence of infection by blood parasites and richness of lineages would differ between the habitats studied. Based on the impact of urbanization on the dynamics of parasite transmissions, we expected different patterns in urban than in natural and rural habitats, due to, for example, a lower density of insect vectors and simplified avian community usually found in more urbanized habitats (Evans et al., 2009b; Ferraguti et al., 2016; Seress and Liker, 2015). In addition, we also expect different patterns of temporal variability in parasite prevalence and richness within different landscape uses, as human activities and urbanization may reduce the environmental variability. Consequently, more urbanized habitats had insect breeding areas that are less influenced by rainfall patterns (Li et al., 2014; Wilke et al., 2019), and host will have more abundant and seasonally stable food from anthropogenic activities that could influence their immune responses against infections (Liker et al., 2008; Salleh Hudin et al., 2016). Finally, distinct patterns may be expected for each of the three parasite genera studied, namely *Plasmodium*, *Haemoproteus* and *Leucocytozoon*, as a consequence of the diverse environmental requirements of the insect groups involved in their transmission (Ciadamidaro et al., 2016; Pérez-Rodríguez et al., 2013; Purse et al., 2015; Santiago-Alarcon and Marzal, 2020; Valkiūnas, 2005).

Materials and Methods

Study area and bird sampling

Fieldwork was conducted in the province of Huelva (southern Spain) from July to October in 2013 and from June to August in 2014, overlapping with the higher density of juvenile house sparrows in the area. Sampling sites included 15 localities grouped into five triplets, each one formed by geographically close localities in an urban (defined as an area with high density of humans), a rural (defined as places with a high density of livestock), and a natural habitat (defined as an area preserved from habitat transformation with lower density of both livestock and humans than the other ones). The categorization of these localities was based on visual characteristics (for further information on the sampling design see Ferraguti et al., 2016). The average distance within localities of the same triplet was 5645.8 m (±SD 4957.5 m). On average, house sparrows have a home range radius of 1000 to 2000 m with only 10% of individuals dispersing over 2000 m (Snow and Perrins, 1998; Summers-Smith, 1963, 1956), which may allow gene flow between these localities. So, in this study we assume that no genetic differences occur between the house sparrows from different localities that may affect their resistance and tolerance to the infection by the studied haemosporidians.

House sparrows were captured using mist-nets and a bird call playback recorder to avoid any potential bias in bird sampling. Birds were ringed with a numbered metal ring, sexed and aged when possible, based on plumage characteristics and skull ossification (Svensson, 2009). In this study, we only analysed juvenile birds (e.g., those born in the calendar year), which were identified based on plumage wearing and/or the absence of complete cranial ossification. The number of recaptured birds between trapping sessions and years was extremely low and thus, only birds from first captures were included in the analyses. A blood sample was extracted from the jugular vein of each bird using sterile syringes. The volume of blood extracted depended on the body size of each bird and never exceeded 1% of their body mass. Blood was stored in Eppendorf tubes, maintained in cold-boxes during the field work, and subsequently kept at 4ºC prior to centrifugation for 10 min at 1700x g (4000 rpm). The cellular fraction was frozen at -20 ºC until further molecular analyses.

Molecular analyses

Genomic DNA was extracted using the Maxwell®16 LEV System Research (Promega, Madison, WI). Unsexed birds using plumage characteristics were molecularly determined following Griffiths et al. (1998, 1996). The infection status and parasite identity was determined through the amplification of a fragment of the cytochrome *b* gene (Hellgren, Waldenström, & Bensch, 2004).

Negative samples were analysed twice to minimize the occurrence of false negatives (McClintock et al., 2010). Both negative controls for PCR reactions (at least one per plate) and DNA extractions (one per 15 samples) were included in the analysis. Positive amplifications were sequenced by Macrogen Inc. (Amsterdam, The Netherlands). Amplicons were sequenced unidirectionally to reduce the economic cost of sequencing a large number of positive reactions (Dubiec et al., 2016). Nevertheless, a number of samples were sequenced bidirectionally with the complementary primer to confirm the identity of lineages present in the area, especially considering novel and uncommon ones (see Supplementary Material, Table S1). Sequences were edited using the software Sequencher™ v 4.9 (Gene Codes Corp. © 1991–2009, Ann Arbor, MI 48108, USA) and the parasite lineages were identified by comparison with sequences deposited in the GenBank (National Center for Biotechnology Information) and MalAvi databases (Bensch et al., 2009). Here, six samples showed evidence of coinfection by the presence of double peaks in the chromatogram, and other six have sequences of low quality. These individuals were only considered in the analyses of parasite prevalence but not for lineage richness. The sequence of the new *Plasmodium* lineage PADOM38 found here was named following the recommendations of Bensch et al., (2009) and deposited in GenBank (accession nº MW264845). Information on bird parasite infections from 2013 was previously analysed in the context of other studies (Ferraguti et al., 2018; Jiménez-Peñuela et al., 2019).

Statistical analysis

The dataset used in this study included 1400 birds (N_{males}=848, $N_{females}$=552). The body condition of birds was estimated following Peig and Green (2009), from body mass and wing length. Unfortunately, the wing length of four individuals was not recorded, so analyses considering body condition were restricted to 1396 birds. Moreover, due to the low quality of the sequences and coinfections, 10 birds infected by *Plasmodium* and 2 birds infected by *Haemoproteus* were not identified to the lineage level and were not include in the analyses of richness, restricting them to 1388 birds.

The effects of landscape use and year on parasite prevalence were tested by fitting Generalized Linear Mixed-Effects Model (GLMM) with a binomial distributed error and a 'logit' link function. The infection status (1: infected, 0: uninfected) of birds, was included as the dependent variable while month of sampling (continuous), body condition (continuous), bird sex (categorical), year of sampling (categorical), habitat (categorical) and the two-way interaction between year and habitat were included as continuous independent variables or fixed factors. Locality nested in triplet was included as a random factor to control for the geographical stratification of the study areas.

To further investigate the variation in parasite prevalence between years and habitat, we fitted Linear Mixed-Effects Models (LMM) by maximum likelihood. We included the prevalence in 2014 at each locality (N=15) as the continuous dependent variable and the prevalence in 2013 (continuous), habitat (categorical) and the two-way interaction between prevalence in 2013 and habitat as independent variables. The triplet was included as a random factor in the analyses. To conduct these analyses, the prevalence of infection for each locality and year was estimated as the least square means for the year and locality interaction, from a Generalized Lineal Model (GLM) with a binomial distributed error including the infection status (1: infected, 0: uninfected) as the dependent variable while controlling for month of sampling, body condition, bird sex, year of sampling and locality (categorical), and the interaction between year and locality. Separate models were fitted for each *Plasmodium*, *Haemoproteus* and *Leucocytozoon*.

The richness of parasite lineages was estimated independently for each genus, year and locality from rarefaction curves with the function *rarefy* (package *vegan*, see below) (Oksanen et al., 2008). This procedure allowed us to control for differences in sample size between localities and years. Subsequently, we used LMM fitted by maximum likelihood to identify the role of landscape use and year on richness. The lineage richness of both years (continuous) (N=30) was included as the dependent variable and year (categorial), habitat (categorical) and their interaction were included as independent variables, and locality was nested into triplet as a random factor. To assess correlation of parasite richness between years, we fitted a model including the lineage richness at each locality in 2014 (continuous) (N=15) as the dependent variable and the lineage richness at each locality in 2013 (continuous), habitat (categorical) and their interaction as independent variables while the triplet was included as a random factor.

Additional Generalized Linear Mixed-Effects Models (GLMM) were fitted to identify potential differences in the distribution of the more common parasite lineages throughout the three habitats. The infection status of birds by each parasite lineage was included as the dependent variable, while year of sampling, habitat and their interaction were included as independent variables. Locality nested into triplet were included as a random factor. Separate models were fitted for each of the most common parasite lineages (i.e., those representing at least 10% of infections by a given parasite genus) while the rest of the lineages and not identified lineages (10 for *Plasmodium* and 2 for *Haemoproteus*) were pooled together in category "others". Models were fitted with a binomial distributed error and a 'logit' link function. We analysed each genus separately, considering only birds infected by *Plasmodium* (N=483), *Haemoproteus* (N=236) and *Leucocytozoon* (N=312). Moreover, heat maps for each year showing the number of birds infected by each parasite lineage in each locality were created using the function *heatmap.2* from the package *ggplot2*.

When we found a significant effect of the variable habitat, differences between the three factor levels were tested with Tukey's Post Hoc Contrasts. In addition, Test contrasts of factor interactions from the package *phia* (Martínez, 2015) were used to test for differences between levels of the interaction between habitat and year. We calculated the variance explained by the models by estimating a *pseudo-*R^2 (Nakagawa and Schielzeth, 2013) using the package MuMin (Bartón, 2019). Collinearity between independent variables was tested with the Variance Inflation Factor (VIF) (Zuur et al., 2010) and we did not find evidence of collinearity (values < 4). Normality of continuous variables and the residuals of all the LMMs were tested by checking normality *qq*-plots and Shapiro-Wilk's normality tests, while overdispersion for GLMMs was checked with Pearson overdispersion test. Variance homogeneity of the variables was tested with a Levene test. The 95% confidence intervals (C.I.) of prevalence estimates were calculated with the function *binconf* from the package *Hmisc*. All the statistical analyses were conducted in R (v, 3.6.0 GUI 1.70 El Capitan build; R Foundation for Statistical Computing 2018) using the packages: *arm*, *car*, *cowplot*, *ggplot2*, *Hmisc*, *lattice*, *lme4*, *lmerTest*, *lsmeans*, *MASS*, *Matrix*, *multcomp*, *MuMIn*, *nlme*, *phia*, *RcppEigen*, *stats*, and *vegan*.

Results

Interannual and landscape variation in parasite prevalence

The 63.6% (C.I.: 59.8-67.1) of the 667 and 58.1% (C.I.: 54.5-61.6) of the 733 birds sampled in 2013 and 2014 respectively, were infected by at least one parasite genus. Of them, 14.4% (C.I.: 11.9-17.3) and 11.0% (C.I.: 8.98-13.5) were coinfections between *Plasmodium* or *Haemoproteus* and *Leucocytozoon* parasites in 2013 and 2014, respectively. *Plasmodium* was the most common parasite found infecting birds (prevalence_{2013}=34.9%, C.I.: 31.4-38.6; prevalence_{2014}=33.7%, C.I.: 30.4-37.2) followed by *Leucocytozoon* (prevalence_{2013}=23.1%, C.I.: 20.0-26.4; prevalence_{2014}=21.5%, C.I.: 18.7-24.7) and *Haemoproteus* (prevalence_{2013}=20.0%, C.I.: 17.1-23.1; prevalence_{2014}=13.9%, C.I.: 11.6-16.6). See Table S1 in Supplementary Material for further details.

In addition, differences between habitats were only significant in 2013, with birds from natural habitats showing higher prevalence of *Leucocytozoon* than rural ones (χ^2=4.56, d.f.=1, p=0.03) (Fig. 1). *Leucocytozoon* prevalence decreased during the sampling period as supported by the negative association between this variable and month (Table 1). The prevalence of *Plasmodium* and *Haemoproteus* was higher in birds with higher body condition while no relationship was found for *Leucocytozoon*. No temporal differences or habitat associations in *Plasmodium* prevalence were found (Fig. 1, Table 1). Variance in parasite prevalence between localities and years was similar between habitats (Levene test, $p > 0.52$ for the three genera). No difference in prevalence was found according to bird sex for any of the three parasites analysed.

Complementarily, we tested the relationships between prevalence in 2014 and in 2013 for each parasite genus. For the case of *Haemoproteus* and *Leucocytozoon*, the prevalence of infection was positively correlated between years. By contrast, the prevalence of infection by *Plasmodium* parasites was not significantly associated between years (Fig. 2, Table 2). Non-significant associations were found for either habitat or the interaction between parasite prevalence during 2013 and habitat for any of the parasite genera, suggesting that the reported associations were independent from the landscape use (Table 2).

Table 1. Results of the GLMM analysing the effects of month of capture, bird sex, body condition, year of sampling, habitat and the interaction between year and habitat on the prevalence of infection by each of the three parasite genera (N=1396). Significant relationships ($p < 0.05$) are highlighted in bold. Conditional (and marginal) R^2 are shown. Est = Estimate; S.E.= Standard Error.

	Plasmodium				***Haemoproteus***				***Leucocytozoon***			
	Est (± S.E.)	**χ^2**	**Df**	***p***	**Est (± S.E.)**	**χ^2**	**Df**	***p***	**Est (± S.E.)**	**χ^2**	**Df**	***p***
Intercept	-3.60 (1.19)	9.20	1	0.002	-4.65 (1.83)	6.46	1	0.01	0.40 (1.52)	0.07	1	0.79
Month	-0.02 (0.11)	0.04	1	0.84	-0.02 (0.15)	0.01	1	0.91	-0.35 (0.14)	6.55	1	**0.01**
Sex: Male	0.00^a	0.01	1	0.91	0.00^a	1.05	1	0.31	0.00^a	0.28	1	0.60
Sex: Female	0.01 (0.12)				-0.17 (0.17)				0.08 (0.15)			
Body condition	0.12 (0.03)	13.70	1	**<0.001**	0.14 (0.05)	9.07	1	**0.003**	0.07 (0.04)	2.96	1	0.09
Year 2013	0.00^a	1.25	1	0.26	0.00^a	4.65	1	**0.03**	0.00^a	8.79	1	**0.003**
Year 2014	0.30 (0.27)				-0.72 (0.33)				-0.90 (0.30)			
Habitat: Natural	0.00^a	1.31	2	0.52	0.00^a	0.93	2	0.63	0.00^a	4.82	2	0.09
Habitat: Rural	0.28 (0.36)				-0.60 (0.95)				-1.63 (0.76)			
Habitat: Urban	-0.10 (0.36)				-0.91 (0.96)				-0.50 (0.75)			
Year*Habitat: 2013*Natural	0.00^a	4.11	2	0.13	0.00^a	3.24	2	0.20	0.00^a	8.11	2	**0.02**
Year*Habitat: 2014*Rural	-0.60 (0.30)				0.64 (0.41)				1.11 (0.41)			
Year*Habitat: 2014*Urban	-0.48 (0.31)				-0.03 (0.40)				0.23 (0.34)			
R^2	0.08 (0.03)				0.41 (0.06)				0.30 (0.05)			

[a] Reference category

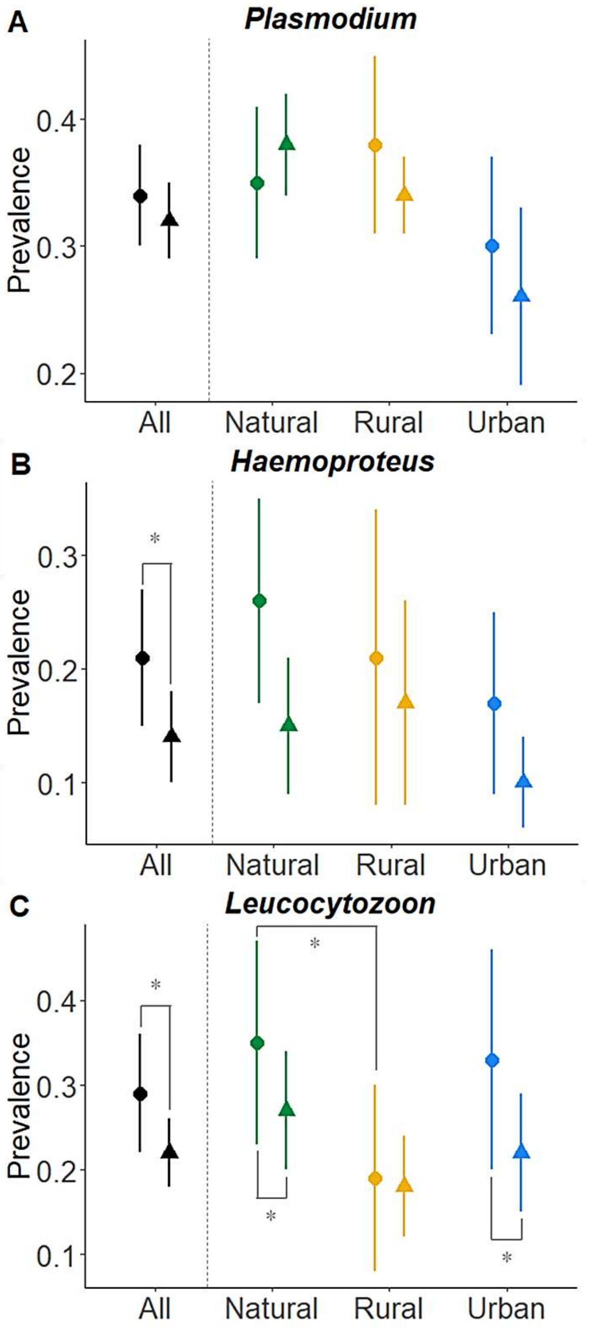

Figure 1. Mean (± standard errors) prevalence of infection by *Plasmodium* (A), *Haemoproteus* (B) and *Leucocytozoon* (C) found in juvenile house sparrows in 2013 (circles) and 2014 (triangles) according to habitat. Account for the different scales of the "y" axe.

Table 2. Results of the LMM analysing the effects of habitat, the prevalence in 2013 and their interaction on the prevalence of infection in 2014 by each of the three parasite genera (N=15). Significant relationships ($p < 0.05$) are highlighted in bold. Conditional (and marginal) R^2 are shown. Est = Estimate; S.E.= Standard Error.

	Plasmodium				***Haemoproteus***				***Leucocytozoon***			
	Est (± S.E.)	**χ^2**	**Df**	***p***	**Est (± S.E.)**	**χ^2**	**Df**	***p***	**Est (± S.E.)**	**χ^2**	**Df**	***p***
Intercept	0.20 (0.16)	1.52	1	0.22	0.01 (0.05)	0.01	1	0.91	0.06 (0.07)	0.86	1	0.35
Prevalence 2013	0.40 (0.40)	0.93	1	0.33	0.70 (0.27)	6.80	1	**0.01**	0.54 (0.17)	9.91	1	**<0.001**
Habitat: Natural	0.00[a]				0.00[a]				0.00[a]			
Habitat: Rural	-0.05 (0.18)	3.16	2	0.21	-0.03 (0.07)	1.29	2	0.53	0.12 (0.10)	2.46	2	0.29
Habitat: Urban	0.31 (0.24)				0.04 (0.06)				-0.04 (0.11)			
Prevalence 2013*Habitat: Natural	0.00[a]				0.00[a]				0.00[a]			
Prevalence 2013*Habitat: Rural	0.04 (0.48)	2.80	2	0.25	0.005 (0.30)	1.01	2	0.60	-0.40 (0.25)	3.90	2	0.14
Prevalence 2013*Habitat: Urban	-0.85 (0.63)				-0.19 (0.30)				0.14 (0.31)			
R^2	0.30 (0.30)				0.79 (0.79)				0.55 (0.55)			

[a] Reference category

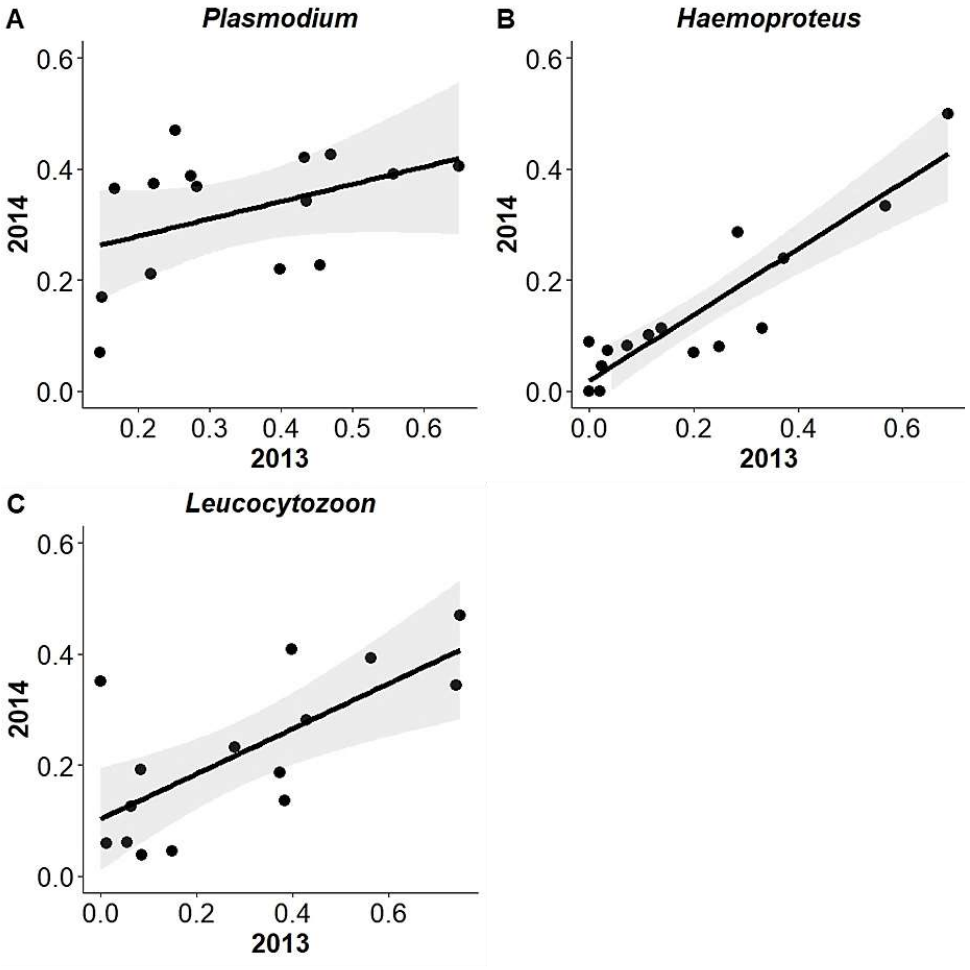

Figure 2. Relationship between the prevalence of infection by *Plasmodium* (A)*,* *Haemoproteus* (B) and *Leucocytozoon* (C), during 2013 and 2014 in wild populations of juvenile house sparrows.

Interannual and landscape variation in parasite lineage richness

Lineage richness did not differ between years for any of the parasites studied. However, lineage richness of *Haemoproteus* showed a significant association with the interaction between year and habitat (Table 3). Only in 2014, a higher *Haemoproteus* richness was found in rural habitats than in natural (χ^2=6.27, d.f.=1, *p*=0.01) and urban ones (χ^2=5.23, d.f.=1, *p*=0.02). In addition, marginally significant differences were found between years only in rural habitats (χ^2=3.41, d.f.=1, *p*=0.06) (Fig. 3). Moreover, a marginally significant effect of the interaction between year and habitat was found for *Leucocytozoon* richness (Table 3) due to significant differences in lineage richness found between years in rural habitats, being higher in 2014 (Fig. 3C; χ^2=7.52, d.f. = 1, *p*=0.006). *Plasmodium* richness was not significantly related to year, habitat nor their interaction (Table 3).

Complementarily, we tested the relationship between the parasite richness in the same localities during the two consecutive years. Surprisingly, overall lineage richness was not correlated between years for any of the parasite tested (Table 4). Further, we found a significant effect of the interaction between *Leucocytozoon* richness during 2013 and the habitat on *Leucocytozoon* richness during 2014 (Table 4). *Leucocytozoon* lineage richness showed a negative correlation between years in rural habitats (Est=-0.87, S.E.= ±0.20, *p*=0.006), but this correlation was not significant in natural (Est=0.32, S.E.= ±0.22, *p*=0.20, Table 4) nor urban habitats (Est=0.16, S.E.= ±0.30, *p*=0.60). Nevertheless, the correlation in lineage richness between years in urban (Est=1.04, S.E.= ±0.29, *p*=0.04) and natural habitats (Est=1.20, S.E.= ±0.29, *p*=0.008) were positive and statistically significant in comparison to rural habitats (Fig. 4, Table 4).

Table 3. Results of the LMM analysing the effects of year, habitat, and their interaction on the richness by each of the three parasite genera (N=30). Significant relationships ($p < 0.05$) are highlighted in bold. Conditional (and marginal) R^2 are shown. Est = Estimate; S.E.= Standard Error.

	Plasmodium				***Haemoproteus***				***Leucocytozoon***			
	Est (± S.E.)	**χ^2**	**Df**	***p***	**Est (± S.E.)**	**χ^2**	**Df**	***p***	**Est (± S.E.)**	**χ^2**	**Df**	***p***
Intercept	2.63 (0.36)	52.91	1	<0.001	1.29 (0.29)	19.77	1	<0.001	1.77 (0.33)	27.91	1	<0.001
Year 2013	0.00[a]	0.27	1	0.61	0.00[a]	1.56	1	0.21	0.00[a]	0.07	1	0.79
Year 2014	0.21 (0.41)				-0.38 (0.31)				0.12 (0.44)			
Habitat: Natural	0.00[a]				0.00[a]				0.00[a]			
Habitat: Rural	0.24 (0.51)	1.64	2	0.44	0.08 (0.41)	0.26	2	0.88	-0.38 (0.47)	1.64	2	0.44
Habitat: Urban	-0.41 (0.51)				0.21 (0.41)				0.22 (0.47)			
Year*Habitat: 2013*Natural	0.00[a]				0.00[a]				0.00[a]			
Year*Habitat: 2014*Rural	-0.10 (0.58)	0.14	2	0.93	0.95 (0.43)	7.33	2	**0.03**	1.09 (0.62)	5.06	2	0.08
Year*Habitat: 2014*Urban	0.12 (0.58)				-0.12 (0.43)				-0.22 (0.62)			
R^2	0.40 (0.09)				0.56 (0.22)				0.30 (0.19)			

[a] Reference category

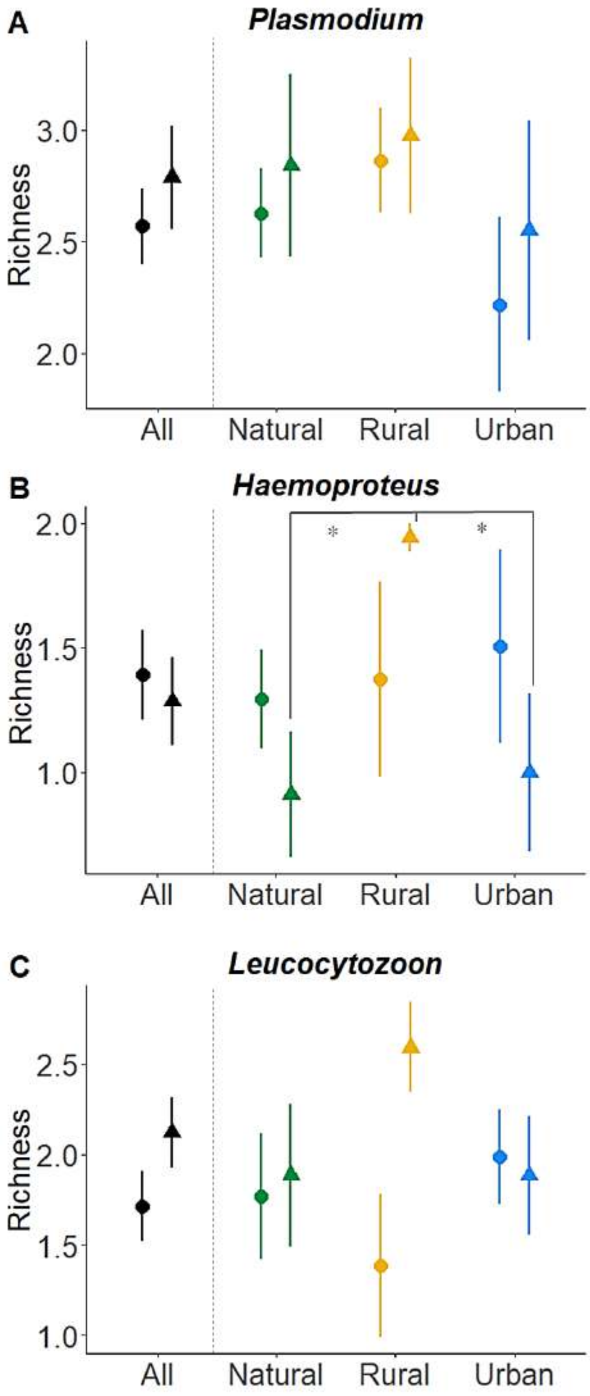

Figure 3. Mean (± standard errors) lineage richness of *Plasmodium* (A), *Haemoproteus* (B) and *Leucocytozoon* (C) found in juvenile house sparrows in 2013 (circles) and 2014 (triangles) according to habitat. Account for the different scales of the "y" axe.

Table 4. Results of the LMM analysing the effects of habitat, the richness in 2013 and their interaction, on the richness in 2014 by each of the three parasite genera (N=15). Significant relationships ($p < 0.05$) are highlighted in bold. Conditional (and marginal) R^2 are shown. Est = Estimate; S.E.= Standard Error.

	Plasmodium				***Haemoproteus***				***Leucocytozoon***			
	Est (± S.E.)	**χ^2**	**Df**	***p***	**Est (± S.E.)**	**χ^2**	**Df**	***p***	**Est (± S.E.)**	**χ^2**	**Df**	***p***
Intercept	0.24 (2.85)	0.007	1	0.93	0.26 (0.68)	0.15	1	0.70	1.31 (0.51)	6.73	1	0.01
Richness 2013	0.99 (1.07)	0.85	1	0.35	0.50 (0.50)	0.99	1	0.32	0.32 (0.22)	2.12	1	0.15
Habitat: Natural	0.00[a]				0.00[a]				0.00[a]			
Habitat: Rural	-0.58 (3.88)	0.84	2	0.66	1.74 (0.79)	12.63	2	**0.002**	2.49 (0.50)	28.20	2	**<0.001**
Habitat: Urban	1.80 (3.12)				-0.27 (0.81)				0.24 (0.73)			
Richness 2013*Habitat: Natural	0.00[a]				0.00[a]				0.00[a]			
Richness 2013*Habitat: Rural	0.17 (1.40)	0.95	2	0.62	-0.55 (0.56)	3.99	2	0.14	-1.20 (0.29)	18.70	2	**<0.001**
Richness 2013*Habitat: Urban	-0.76 (1.20)				0.17 (0.56)				-0.16 (0.37)			
R^2	0.20 (0.18)				0.63 (0.63)				0.90 (0.38)			

[a] Reference category

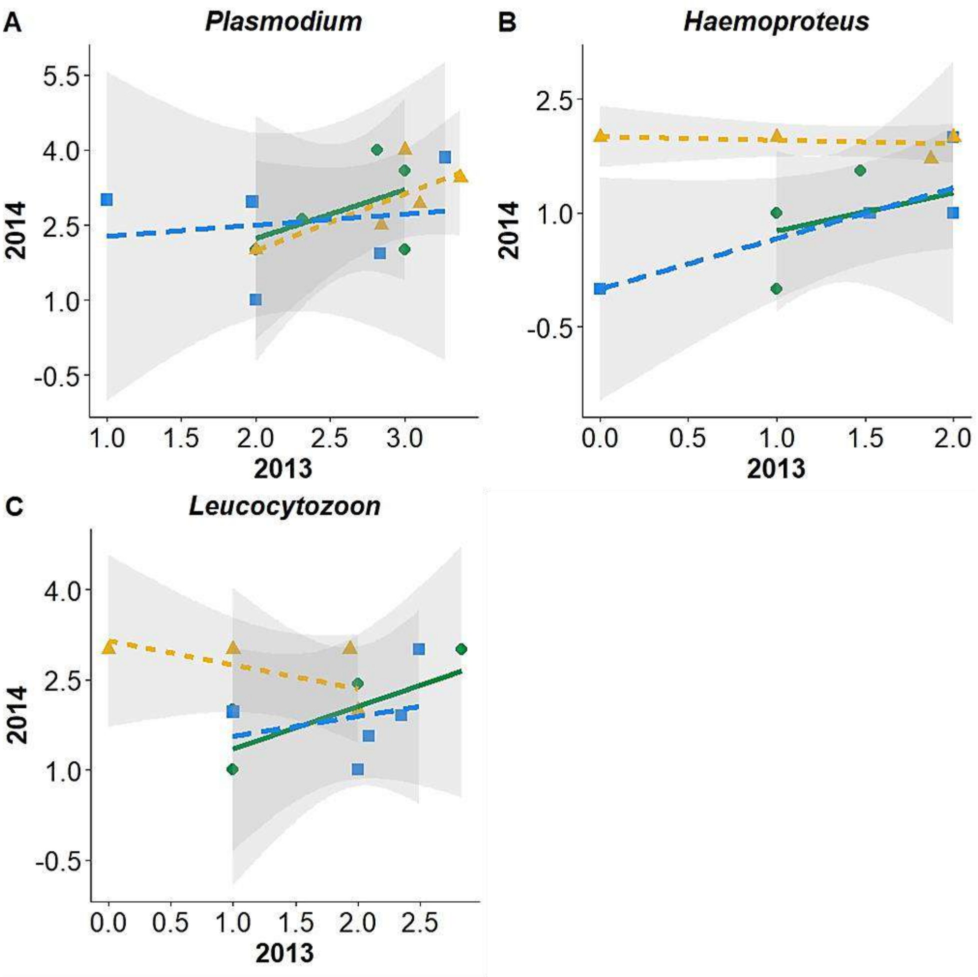

Figure 4. Relationship between lineage richness of *Plasmodium* (A)*, Haemoproteus* (B)*, and Leucocytozoon* (C) parasites between 2013 and 2014 according to the different habitat. Natural, rural, and urban habitats are shown in green straight line with circles, yellow small-dotted line with triangles and blue big-dotted line with squares, respectively.

Interannual and landscape variation in specific parasite lineages prevalence

We found 23 different parasite lineages infecting house sparrows, including 13 lineages of *Plasmodium* spp., 4 lineages of *Haemoproteus* spp. and 6 lineages of *Leucocytozoon* spp. (see Table S2 in Supplementary Material). The distribution of the different parasite lineages was homogeneous among and between habitats, with one linage per genus harbouring the most part of infected birds: SGS1 for *Plasmodium*, PADOM05 for *Haemoproteus* and RECOB3 for *Leucocytozoon* (Fig. 5). Moreover, the analyses revealed that the incidence of the most common parasite lineages (i.e., those representing more than 10% of infections) were not significantly associated with year of sampling, habitat, or their interaction. Similar conclusions were obtained in analyses where we pooled the remaining lineages of each genus (Tables S3, S4 and S5).

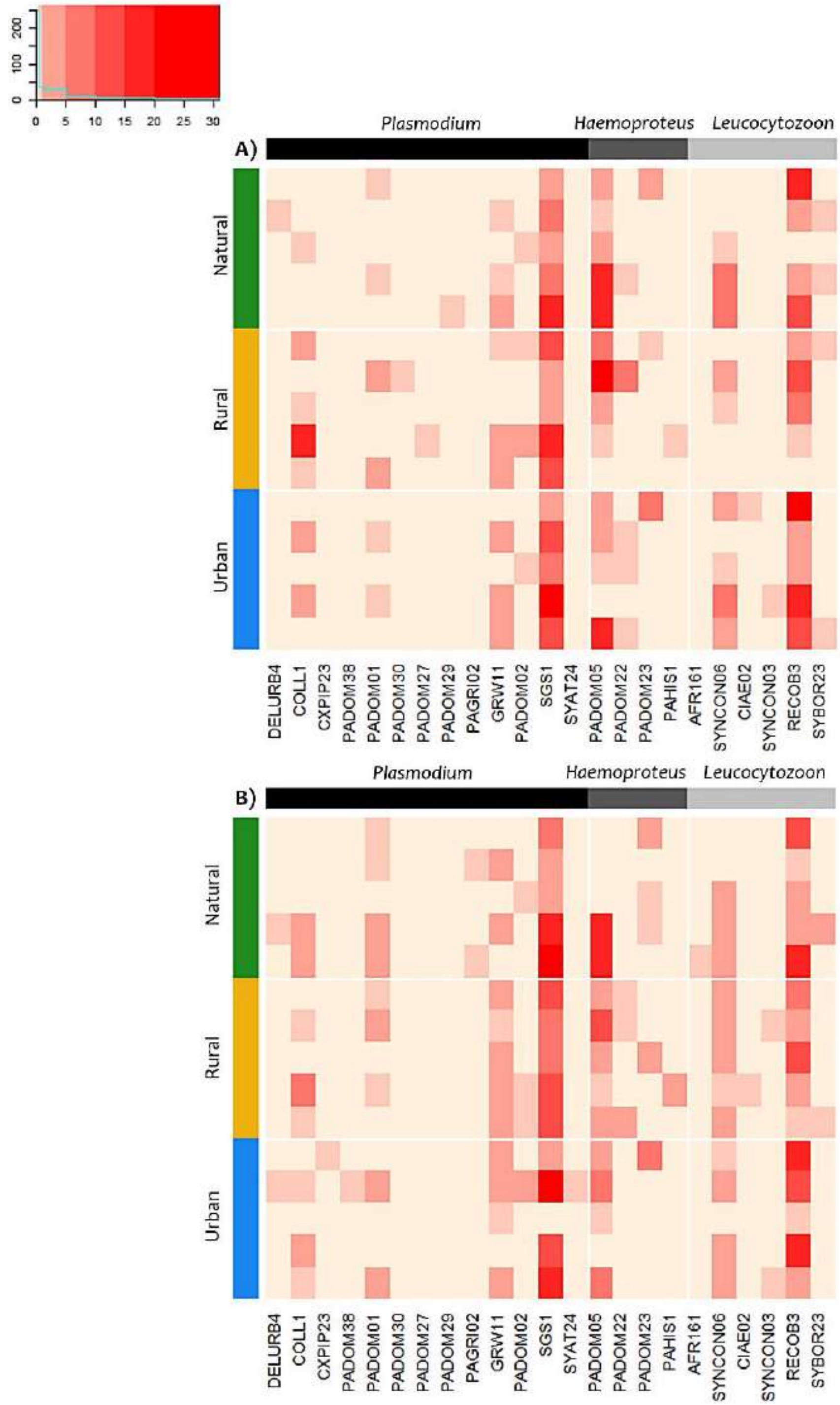

Figure 5. Heat map of the number of house sparrows found infected by each lineage of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* throughout the different habitat in 2013 (A) and 2014 (B). Lineages were named according to MalAvi.

Discussion

According to our results, the prevalence of infection by both *Haemoproteus* and *Leucocytozoon* in house sparrows was higher in 2013 than in 2014, particularly for *Leucocytozoon*, in urban and natural habitats. Also, *Leucocytozoon* prevalence was higher in natural habitats than in rural ones for the first year. The prevalence of *Plasmodium* and *Haemoproteus* was higher in birds with higher body condition and *Leucocytozoon* prevalence decreased along the month of sampling. The prevalence of infection by both *Haemoproteus* and *Leucocytozoon* at population level, showed a positive association between years independently of the habitat. Moreover, birds from rural habitats harboured higher *Haemoproteus* lineage richness compared to urban and natural habitats during 2014. Additionally, *Leucocytozoon* lineage richness showed negative and significant correlations between years in rural habitats and a positive correlation in urban and natural habitats in comparison. No significant associations of year, habitat or its interaction were found for *Plasmodium* prevalence nor richness. These results certainly support that in spite of the similarities in the life-cycle of the three parasite genera studied here, clear differences could be found according to the host-parasite system considered, as previously reported (Calegaro-Marques and Amato, 2014; Hernández-Lara et al., 2017; Pérez-Rodríguez et al., 2013; Turcotte et al., 2018).

Biting midges (*Culicoides*) are the main vectors of *Haemoproteus* (subgenus *Parahaemoproteus*), blackflies (*Simuliidae*) transmit *Leucocytozoon* parasites, and mosquitoes (*Culicidae*) are the vectors of *Plasmodium* parasites (Atkinson and Van Riper, 1991; Valkiūnas, 2005). The prevalence of vector-borne parasites, as those studied here, are strongly determined by the abundance and diversity of insect vectors in the area (Lalubin et al., 2013; Martínez-de la Puente et al., 2013). Indeed, according to previous modelling studies, an increase in vector diversity may facilitate pathogen transmission success (Roche et al., 2013, 2012) by favouring the probability that pathogens will interact with competent vectors. Therefore, differences in the environmental requirements between vector groups may determine the differential epidemiology of these parasites, with environmental and climatic factors such as temperature, rainfall or drought, playing a major role on vector populations (Patz et al., 2008). Thus, the interannual differences in *Haemoproteus* and *Leucocytozoon* prevalence found in this study could be due to differential weather conditions between years that directly affect the abundance of biting midges and black flies in the area. Our results agree with other studies where authors sampled birds in different years and also found differences in the prevalence of blood parasites (Bensch et al., 2007; Dubiec et al., 2016; Geue and Partecke, 2008; Knowles et al., 2011). Moreover, weather conditions strongly affect the numbers of blood-sucking arthropods feeding on birds since their earlier stage of development (Martínez-de la Puente et al., 2009; Merino and Potti, 1996), potentially affecting their exposure to haemosporidians. The reason why *Leucocytozoon* show interannual differences in prevalence only in urban and natural habitats but not in rural ones, might be determined by specific habitat characteristics. Certainly, apart from weather conditions, environmental features in the area, including the availability of breeding areas for insect vectors (Becker et al., 2010), may determine stability in parasite prevalence. Biting midges require soil-water interface spaces for breeding (Purse et al., 2015) and blackflies breeds in constant running water bodies (Ciadamidaro et al., 2016). Thus, the positive relationship in the prevalence of infection between years found for *Haemoproteus* and *Leucocytozoon* suggest that populations of both biting midges and blackflies may found stable environmental characteristics (e.g., breeding sites). This may allow the reproduction of these vectors in the studied localities during the two consecutive years despite the weather conditions variations that cause the differences found between years. Moreover, the availability of running water bodies may decrease along the season with the increment of temperature and decrease of precipitation, which could explain, at least in part, the decrease in the *Leucocytozoon* prevalence along the sampling period. By contrast, mosquitoes use different breeding environments with most species thriving in small temporary water bodies, which make them largely dependent of precipitation and temperatures of the year (Becker et al., 2010). Weather conditions in Mediterranean areas highly varies between years and seasons in terms of precipitations, drastically varying the populations of avian *Plasmodium* vectors, including the species *Culex pipiens* (Roiz et al., 2014). Due to the key role of *Culex* species such as *Cx. pipiens* in the transmission of avian *Plasmodium* (Ferraguti et al., 2020; Gutiérrez-López et al., 2020), fluctuating environmental conditions affecting their populations may explain, at least in part, the absence of interannual differences or correlation in the prevalence of *Plasmodium* parasites (Gangoso et al., 2020).

In addition, we cannot rule out the possibility that other factors may also explain the pattern of infections found in wild birds. For instance, food availability in terms of abundance, quality and stability allows birds to invest more resources on immune defence (Houston et al., 2007). Immune responses are energetically costly, so those individuals in better body condition may be capable of producing stronger immune responses (Navarro et al., 2003). Also, even if abundant, anthropogenic food is usually rich in carbohydrates and fats but low in protein content, and consequently may now allow to fulfil all physiological requirements when consumed by wild birds (Chamberlain et al., 2009; Meyrier et al., 2017; Seress et al., 2019). Our results show that birds infected by *Plasmodium* or *Haemoproteus* had higher body condition, as was previously recorded in a prior study conducted in the area (Jiménez-Peñuela et al., 2019). These results could be explained based on a selective mortality of infected birds showing a poorer body condition, unable to fought off infection. Additional factors could also explain our results. Potential genetic differences (i.e., MHC genes) in birds from different areas could explain their differential capacity to respond against parasites (Bichet et al., 2015) as particular haplotypes may confer both susceptibility or resistance to blood parasite infections (Rivero-de Aguilar et al., 2016). We do not have information on the gene flow between localities to test this possibility, however, house sparrows have a home range radius of 1000 to

2000 m with the 10% of individuals dispersing over 2000 m (Snow and Perrins, 1998; Summers-Smith, 1963, 1956) which may allow the genetic flow between birds populations of the localities of study and reduce potential genetic differences. Finally, we cannot exclude either the possibility that parasite virulence varies between parasite lineages in addition to parasite genera (Lachish et al., 2011b), thus explaining the patterns of infection found here. Nevertheless, the different lineages identified in house sparrows from this study showed a generalist distribution, without significant differences according to year nor habitat.

Interestingly, we found significant differences in the richness of *Haemoproteus* lineages between habitats only in 2014, being rural habitats richer than urban and natural ones. In addition, marginal differences in the *Leucocytozoon* lineage richness between years were only found in rural habitats. Our results show that the differences in lineage richness were mainly driven by changes in rural localities from 2013 to 2014. Specifically, there is a rural locality without *Haemoproteus* nor *Leucocytozoon* infections in 2013, which presents low prevalence in 2014 by at least two *Haemoproteus* and three *Leucocytozoon* lineages (Fig. 5). This pattern may be the result of an increase in *Haemoproteus* and *Leucocytozoon* vector abundance (biting midges and black flies) in this rural locality favouring the circulation of both genera in the area during 2014. In addition, the richness of parasite lineages infecting birds is potentially determined by the host community in the area, which may influence the range of circulating parasites able to infect a target host species (Ellis et al., 2020; Ferraguti et al., 2018; Lachish et al., 2013; Ricklefs et al., 2005, 2004; Sehgal, 2010; Wood et al., 2007). The composition and relative density of species in a bird community are highly influenced by landscape alterations and anthropogenic provision of food influences the size, structure and composition of bird populations (Clark et al., 2018; Evans et al., 2009b; Shochat et al., 2006). Besides, although variable between parasite lineages and species, parasite can cover different host ranges. *Plasmodium* are considered generalist parasites compared with *Haemoproteus* or

Leucocytozoon, which tend to be found in more specialized range of avian species (Beadell et al., 2006; Bensch et al., 2009; Clark et al., 2018, 2014; Hellgren et al., 2008; Valkiūnas, 2005). For this reason, we cannot exclude the possibility that the changes in lineage richness could reflect differences in host community composition, or the circulation of lineages previously absent in the locality. Nevertheless, analyses on the specific lineages found in birds from different habitats support that parasite linages were homogeneously distributed among and between habitats, so no particular associations between lineages and habitat types were observed.

Conclusions

To sum up, our results support the effects of anthropogenic alterations of the landscape on the prevalence and lineage richness of two avian haemosporidian genera, *Haemoproteus* and *Leucocytozoon*, and their temporal variation dynamics through two consecutive years. The different patterns found for each parasite genera highlight the importance of evaluating specific parasite-host-vector systems separately, as their response could differ between genera even under the same scenarios. Moreover, longitudinal studies on the patterns of infections in birds from different habitats are essential to identify their temporal variation. This is especially the case of areas highly influenced by human activities which may determine, directly or indirectly, the community composition of both vectors and hosts populations as well as the health status of birds in these areas. Our results suggest that changes in the community of insect vectors, driven both by the availability of reproductive areas and climate conditions, may highly influence the dynamics of parasite prevalence and lineage richness in the host populations. Besides, our results bring out the importance of considering not only areas with different degrees of anthropization, including cities or towns of different sizes, but also areas where humans develop a variety of activities (i.e., farming).

Ethic statement

Bird trapping was carried out with all the necessary permits from Consejería de Medio Ambiente, and Consejería de Agricultura, Pesca y Desarrollo Rural (Junta de Andalucía) and bird sampling on private land and in private residential areas were conducted with all the necessary permits and consent from the owners. The CSIC Ethics Committee approved the experimental procedures on 9 March 2012.

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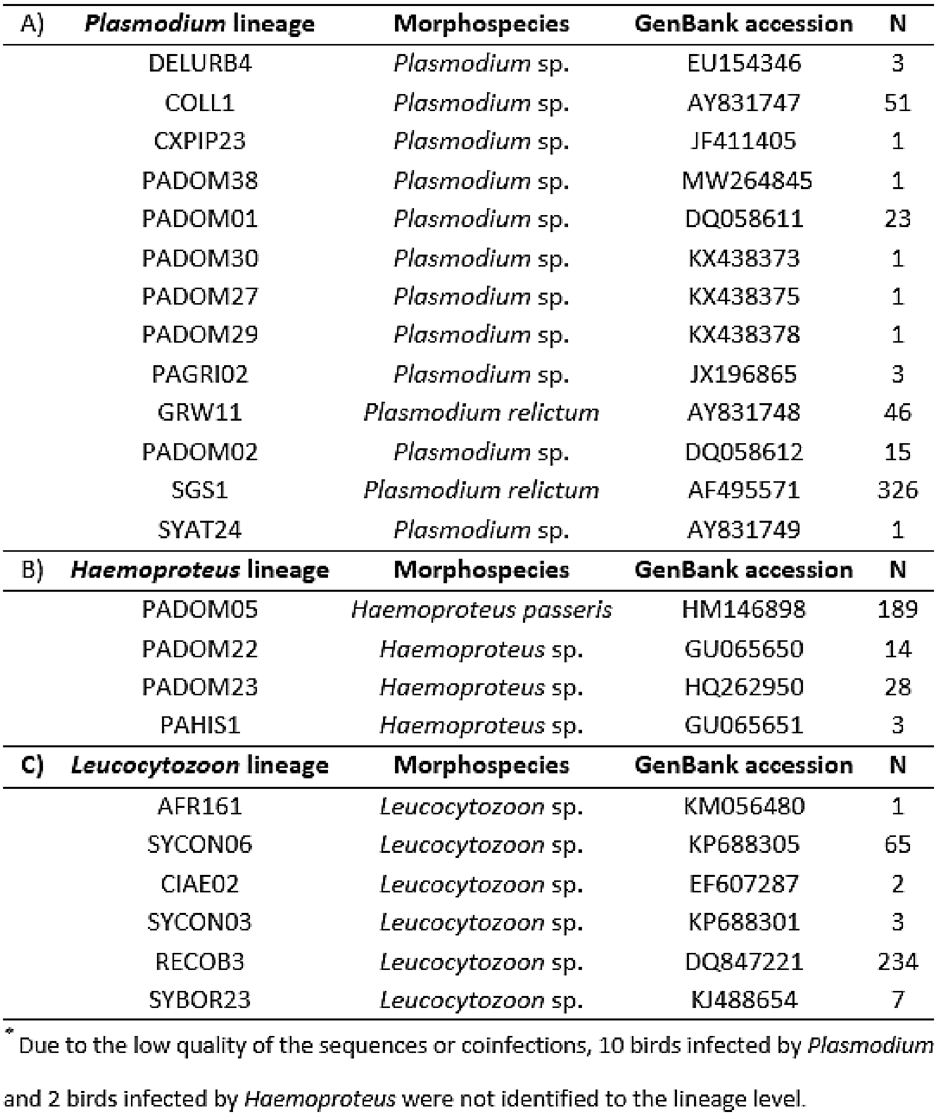
Supplementary material

Table S1. Number of juvenile house sparrows used in the estimation of the prevalence and richness, parasite prevalence (back transformed from estimated least square means), and lineage richness (estimated from a rarefaction curve) for *Plasmodium*, *Haemoproteus* and *Leucocytozoon* for each habitat category of the 15 localities studied.

				Plasmodium				***Haemoproteus***				***Leucocytozoon***			
		N		**Prevalence**		**Richness**		**Prevalence**		**Richness**		**Prevalence**		**Richness**	
Triplet	**Habitat**	**2013**	**2014**	**2013**	**2014**	**2013**	**2014**	**2013**	**2014**	**2013**	**2014**	**2013**	**2014**	**2013**	**2014**
Doñana	Natural	11	12	0.56	0.41	3.00	2.00	0.15	0.07	1.00	1.00	0.13	0.35	1.00	2.00
Doñana	Rural	75	62	0.56	0.38	3.37	3.45	0.02	0.04	2.00	2.00	0.01	0.06	1.00	3.00
Doñana	Urban	70	54	0.46	0.23	2.84	1.93	0.00	0.00	0.00	0.00	0.4	0.41	2.35	1.91
Beas	Natural	36	56	0.25	0.46	3.00	3.58	0.57	0.32	1.48	1.56	0.37	0.18	2.83	3.00
Beas	Rural	51	26	0.18	0.37	3.00	4.00	0.7	0.5	1.87	1.71	0.28	0.23	1.93	3.00
Beas	Urban	52	61	0.29	0.37	1.98	2.96	0.36	0.11	1.53	1.00	0.39	0.14	2.49	3.00
Gibraleón	Natural	23	32	0.23	0.23	2.00	2.00	0.27	0.09	2.00	1.00	0.75	0.47	1.00	1.00
Gibraleón	Rural	12	40	0.23	0.36	2.00	2.00	0.14	0.11	1.00	2.00	0.57	0.39	2.00	1.96
Gibraleón	Urban	32	48	0.16	0.16	1.00	3.00	0.41	0.24	2.00	2.00	0.75	0.36	2.09	1.56
Huelva	Natural	40	25	0.27	0.37	2.82	4.00	0.02	0.00	1.00	0.00	0.09	0.04	2.00	1.00
Huelva	Rural	54	85	0.41	0.22	3.10	2.93	0.00	0.09	0.00	2.00	0.00	0.04	0.00	3.00
Huelva	Urban	68	16	0.15	0.07	2.00	1.00	0.04	0.08	2.00	1.00	0.05	0.06	2.00	1.00
San Juan del Puerto	Natural	52	75	0.43	0.41	2.31	2.62	0.29	0.27	1.00	1.00	0.43	0.28	2.00	2.42
San Juan del Puerto	Rural	43	49	0.44	0.33	2.84	2.50	0.12	0.09	2.00	2.00	0.09	0.19	2.00	2.00
San Juan del Puerto	Urban	48	92	0.49	0.43	3.27	3.85	0.08	0.08	2.00	1.00	0.06	0.13	1.00	1.96

Table S2. Number of juvenile house sparrows found infected by each lineage (N) of *Plasmodium**, *Haemoproteus** and *Leucocytozoon*. Lineages were named according to MalAvi.



A)	***Plasmodium* lineage**	**Morphospecies**	**GenBank accession**	**N**
	DELURB4	*Plasmodium* sp.	EU154346	3
	COLL1	*Plasmodium* sp.	AY831747	51
	CXPIP23	*Plasmodium* sp.	JF411405	1
	PADOM38	*Plasmodium* sp.	MW264845	1
	PADOM01	*Plasmodium* sp.	DQ058611	23
	PADOM30	*Plasmodium* sp.	KX438373	1
	PADOM27	*Plasmodium* sp.	KX438375	1
	PADOM29	*Plasmodium* sp.	KX438378	1
	PAGRI02	*Plasmodium* sp.	JX196865	3
	GRW11	*Plasmodium relictum*	AY831748	46
	PADOM02	*Plasmodium* sp.	DQ058612	15
	SGS1	*Plasmodium relictum*	AF495571	326
	SYAT24	*Plasmodium* sp.	AY831749	1
B)	***Haemoproteus* lineage**	**Morphospecies**	**GenBank accession**	**N**
	PADOM05	*Haemoproteus passeris*	HM146898	189
	PADOM22	*Haemoproteus* sp.	GU065650	14
	PADOM23	*Haemoproteus* sp.	HQ262950	28
	PAHIS1	*Haemoproteus* sp.	GU065651	3
C)	***Leucocytozoon* lineage**	**Morphospecies**	**GenBank accession**	**N**
	AFR161	*Leucocytozoon* sp.	KM056480	1
	SYCON06	*Leucocytozoon* sp.	KP688305	65
	CIAE02	*Leucocytozoon* sp.	EF607287	2
	SYCON03	*Leucocytozoon* sp.	KP688301	3
	RECOB3	*Leucocytozoon* sp.	DQ847221	234
	SYBOR23	*Leucocytozoon* sp.	KJ488654	7

* Due to the low quality of the sequences or coinfections, 10 birds infected by *Plasmodium* and 2 birds infected by *Haemoproteus* were not identified to the lineage level.

SECTION 2

WILD BIRDS HEALTH

CHAPTER 2

URBANIZATION AND BLOOD PARASITE INFECTIONS AFFECT THE BODY CONDITION OF WILD BIRDS

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Graphical abstract

Highlights:

- The joint effect of urbanization and parasite infection on birds was investigated.
- 45 populations of house sparrow from Spain in an urbanization gradient were studied.
- Bird body mass decreased along the urbanization level, especially in urban areas.
- *Plasmodium* and *Haemoproteus* infections were positively associated with body mass.
- Selective mortality may explain the higher body mass of urban infected birds.

Keywords: avian malaria parasites, body mass, house sparrows, *Passer domesticus*, synergic effects, yearlings.

Abstract

Human landscape transformation, especially urbanization, strongly affects ecosystems worldwide. Both urban stressors and parasites have negative effects on organism health, however the potential synergy between those factors has been poorly investigated. We analysed the body condition (i.e. body mass after controlling for wing chord) of 2,043 house sparrows (adults and yearlings) captured in 45 localities along an urbanization gradient in relation to *Plasmodium*, *Haemoproteus* and *Leucocytozoon* infection status. Body condition was negatively related to urbanization level and to urbanized land coverage but only in yearling birds from urban habitats. In addition, bird body condition tended to increase in rural habitats, significantly in the case of yearlings. Infected individuals by *Plasmodium* or *Haemoproteus* had higher body condition than un-infected birds, but this pattern could be due to a selective disappearance of infected individuals with lower body condition as suggested by the reduced variance in body condition in infected birds in urban habitats. These results provide support for a negative impact of urbanization on bird body condition, while *Plasmodium* and *Haemoproteus* may exert selection against individuals with lower body condition living in urban habitats, especially during earlier life stages, underlining the synergistic effects that urbanization and parasites may have on wild birds.

Introduction

The urbanization process implies important landscape transformation and environmental impacts that widely exceed the geographic limits of cities (Bradley and Altizer, 2007; Grimm et al., 2000). Urbanization is usually associated with a general loss of biodiversity (Seress and Liker, 2015) creating altered environments where only a reduced number of species are capable to survive (Mckinney, 2002; Sol et al., 2014). Most avian species have a low tolerance to urbanization suffering important costs in body size and condition (Liker et al., 2008). Survival, infection levels, oxidative status, stress levels and colouration are common animal life-history traits affected by urbanization (Sepp et al., 2018).

Different factors may explain the impact of urbanization on wildlife populations. For instance, food resources provided by humans in urban habitats are rich in carbohydrates and fats, and poor in protein content compared to natural food (Heiss et al., 2009; Meyrier et al., 2017) which may compromise avian reproductive success (Chamberlain et al., 2009; Pollock et al., 2017; Sumasgutner et al., 2014). This can affect population demography (Salleh Hudin et al., 2016) even for omnivorous species able to feed on human waste (Meyrier et al., 2017). Furthermore, urban habitats are polluted with toxic chemicals such as heavy metal particles, gases and other substances (Herrera-Dueñas et al., 2014). These urban pollutants cause oxidative damage as well as long and short-term stress imposing deleterious effects on wild bird populations (Bauerová et al., 2017; Herrera-Dueñas et al., 2014; Isaksson, 2015; Salmón et al., 2016; Watson et al., 2017). Birds from urban populations have a higher telomere shortening rate and higher expression of genes related to immune, inflammatory and oxidative stress responses than individuals living in rural populations (Salmón et al., 2016; Watson et al., 2017). Additionally, Bailly et al. (2016) experimentally demonstrated that the activation of constitutive immunity was more costly in urban great tits (*Parus major*), as confirmed by a decrease in haptoglobin production and a loss of body mass.

However, other species are capable of thriving in such urban environments reaching high abundances in these areas (Meyrier et al., 2017; Seress and Liker, 2015). The potential benefits provided by urban habitats include milder climate (Saaroni et al., 2000), higher predictability of food and water (Fokidis et al., 2008) and higher availability of nesting sites through nest boxes or buildings (Sumasgutner et al., 2014). Overall, these factors can improve the health status and survival of birds (Chamberlain et al., 2009; Dulisz et al., 2016), or at least buffer the negative impact of urbanization (Sumasgutner et al., 2014). Thus, urban habitats could represent ecological traps for wildlife due to mismatches between animal perception of attractive urban features and the cost imposed on fitness (Meyrier et al., 2017; Pollock et al., 2017; Sumasgutner et al., 2014). Therefore, it is essential to identify the factors affecting urban wildlife and determine their consequences for individuals living in these habitats (Seress and Liker, 2015).

Urbanization may influence pathogen epidemiology and host susceptibility to infectious diseases (Bradley and Altizer, 2007). Although the effects of urban areas may differ when the host-parasite assemblage is considered (Delgado-V. and French, 2012), landscape disturbance is expected to affect the dynamics of vector-borne parasites (Calegaro-Marques and Amato, 2014; Ferraguti et al., 2018). However, analyses of the urbanization effect on the prevalence and impact of pathogen infections have provided contradictory results. While the severity of infections by *Plasmodium*, poxvirus and coccidians in birds increased with urbanization (Bichet et al., 2013; Giraudeau et al., 2014), a decrease was found for the case of infection by different blood parasites (Fokidis et al. 2008; Geue and Partecke 2008).

The avian malaria parasites of the genus *Plasmodium* and the related genera *Haemoproteus* and *Leucocytozoon*, are common vector-borne parasites infecting birds (Atkinson and Van Riper III, 1991; Valkiũnas, 2004). Experimental and correlative studies have provided support for the impact of blood parasite infections on bird health (Valkiũnas et al., 2006), reproductive success (Marzal et al., 2005; Merino et al., 2000), susceptibility to predation (García-Longoria et al., 2015; Møller and Nielsen, 2007) and life expectance (Martínez-de la Puente et al., 2010; Marzal et al., 2008). Immune responses to fight off infections by blood parasites are costly, with only those individuals in better body condition being able to develop more effective responses (Navarro et al., 2003). A diet of bad quality or quantity, as may be the case in birds living in urban habitats, may increase host susceptibility to infectious diseases (Cornet et al., 2014).

Here, we assess the impact of both urbanization and blood parasite infection on the body condition (i.e. body mass after controlling for wing chord) of wild house sparrows (*Passer domesticus*) through an urbanization gradient in southern Spain. We sampled birds in habitats with different landscape use, from urban habitats to rural and natural environments. We selected house sparrows as the model species because this bird is ubiquitous, sedentary and abundant throughout most of their distribution range, especially when linked to human settlements (SÆtre et al., 2012; Seress and Liker, 2015). House sparrows are also found in natural habitats although this species usually occurs within or nearby human constructions, being uncommon in forest habitats, scrublands and wetlands (Bichet et al., 2014; BirdLife International, 2017). This species is considered the best example of an urban exploiter (Meillère et al., 2015), even though it is currently undergoing consistent population decline across Europe (De Laet and Summers-Smith, 2007). House sparrows are commonly infected by haemosporidian parasites (Bichet et al., 2013), and at least 49 different genetic lineages of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* have been recorded in this species (according to MalAvi, accessed 24 September 2018, Bensch, Hellgren, & Pérez-Tris, 2009). Thus, this species represents a suitable study model to assess the impact of urbanization and parasite infection on individual traits (Liker et al., 2008). Based on previous studies on passerines (Isaksson, 2015; Sepp et al., 2018), we predict a negative impact of urban environments on house sparrow body condition, particularly in those individuals infected by avian haemosporidians.

Material and methods

Study area and bird sampling

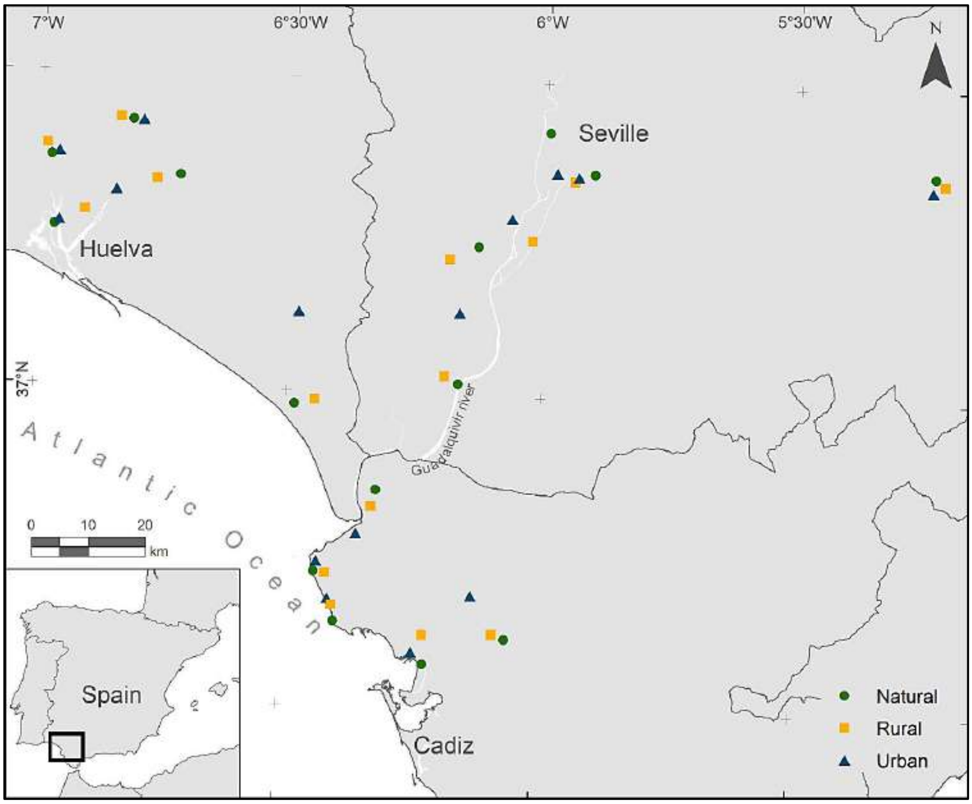


Figure 1. Distribution of the 45 sampling sites (localities) grouped in 15 triplets of urban (blue triangle), rural (yellow square) and natural (green point) habitats from southern Spain.

Field work was conducted at 45 localities in the provinces of Cadiz (n=15), Huelva (n=15) and Seville (n=15), southern Spain (Fig.1). These localities were geographically grouped into triplets formed by one urban (with a high human population density), one rural (with a high density of livestock) and one natural habitat (a better conserved region where wildlife predominated in comparison to livestock and human populations). Human density (people/250m^2) differed between habitats (χ^2=26.72; d.f.=2; p<0.001) with urban areas being more densely populated (mean population= 187.41) than both rural (mean population= 11.70; Tukey post hoc test; p < 0.001) and natural (mean population= 5.47; p < 0.001) ones. The average distance within localities of the same triplet was 5,739.87 meters (±SD 4,465.12 meters), which minimize the possibility of individuals moving from one habitat to another. House sparrows have a home range radius of 1,000 to 2,000 m with less than 10% of individuals dispersing over 2,000 m (Snow and Perrins, 1998; Summers-Smith, 1963, 1956).

Wild house sparrows were sampled in these localities from July to October 2013 by three teams of specialized ringers. Birds were captured using mist-nets and a bird call playback recorded, which was used every single time to minimize potential bias in bird sampling (Figuerola and Gustamante, 1995). Each bird was ringed with a numbered metal ring, and its age and sex were determined when possible, according to their plumage characteristics and skull ossification (Svensson, 2009). Birds were identified as yearlings (birds younger than one year, Euring code 3) or adults (birds older than one year, Euring code 4). Those birds identified as unknown age (Euring code 2) were excluded from the analyses (N=271 in this study). Body mass of each bird was measured with an electric balance (to the nearest 0.1 g) and wing chord was measured with a metal ruler (to the nearest 1 mm) by the same ringer in each tripled of the same province. Both, wing chord (r = 99.93) and body mass (r = 99.99) were highly repeatable measurements between observers as estimated from 12 individuals measured. Finally, a blood sample was obtained from the jugular vein using a sterile syringe, never exceeding 1% of the body mass. Blood samples were transferred to Eppendorf tubes and preserved in cold-boxes during the field work. In the laboratory, the samples were kept at 4ºC up to 24 hours and subsequently centrifuged for 10 minutes at 1,700 g (4,000 rpm) to separate serum and cellular fractions, which were frozen at -80 or -20ºC, respectively.

Molecular analyses

Genomic DNA was extracted from the blood cellular fraction using Maxwell®16 LEV System Research (Promega, Madison, WI) (Gutiérrez-López et al., 2015). For those birds which were not sexed based on morphology (mainly juveniles), bird DNA was used to molecularly sex birds following Griffiths et al. (1998, 1996). In addition, the infection status by *Plasmodium*, *Haemoproteus* and *Leucocytozoon* was assessed following the protocol detailed by Hellgren et al. (2004). PCR of samples providing negative results (absence of parasite DNA) were repeated to avoid the occurrence of false negatives (McClintock et al., 2010). Both negative controls for PCR reactions (at least one per plate) and DNA extraction (one per 15 samples) were included in the analysis. PCR amplifications were resolved in 1.2% agarose gels and positive samples were sequenced uni-directionally from the 5′ end using the Macrogen sequencing service (Macrogen Inc., Amsterdam, The Netherlands). Amplicons were sequenced uni-directionally to reduce the cost of sequencing a large number of amplicons (Dubiec et al., 2016). Sequences were edited using the software Sequencher™ v 4.9 (Gene Codes Corp. © 1991–2009, Ann Arbor, MI 48108, USA) and parasite genera were identified by BLAST comparison to previously identified sequences deposited in the GenBank database (National Center for Biotechnology Information).

Landscape characterization

Urbanization scores of the 45 sampling localities were obtained following Czúni et al. (2012), Seress et al. (2014) and Lipovits et al. (2015) (http://keplab.mik.uni-pannon.hu/en/urbanization-index). Satellite images taken in 2013 (the year of bird sampling) were obtained for the 45 localities from Google Maps in '.jpeg' format. Satellite images covered an area of 1 square km around each bird sampling locality. For each square, the software estimates the proportion of buildings, vegetation and paved surface coverture, which are then combined in a Principal Components Analysis (PCA) to obtain an 'urbanization score'. The urbanization score ranges from negative values for the case of less urbanized areas to higher values for more intensely urbanized areas. Moreover, to obtain a more complete estimation of the environmental characteristics of the sampling localities, the percentage of the total area occupied by agricultural, natural and urban lands was characterized (see details in Ferraguti et al. (2016)). To do that, we used the cartography from the study area (http://www.juntadeandalucia.es/institutodeestadisticaycartografia/DERA/) considering a 2,000 meter radius buffer around the bird sampling sites, covering approximately the range of dispersal of adult house sparrows (Snow and Perrins, 1998). Spatial analyses and zonal statistical tools for raster files and the geoprocessing intersect tool for vector variables were used with the software ArcGis v10.2.1 (ESRI, Redlnad). The land surface covered by forests were not considered in our analyses as house sparrows are commonly absent or very uncommon in most of these habitats (Anderson, 2006). Finally, human population density was estimated as the number of people living in a grid of 250 x 250 meters according to the data registered on 1st January 2013 by the Institute of Statistics and Cartography of Andalusia.

Statistical analyses

Statistical analyses were conducted using Linear Mixed-Effects Models (LMM) in R (v, 3.5.1.; R Foundation for Statistical Computing 2018) using the packages: *MASS*, *car*, *lattice*, *Matrix*, *Rcpp*, *lme4*, *MuMIn*, *arm*, *stats*, *multcomp*, *ggplot2*, *nlme* and *scales* (Crawley, 2007). Dependent and independent variables and the residuals of the models were checked for normality with *qq* plots. Also, we checked for collinearity in independent variables by calculating their Variance Inflation Factor (VIF) in all models, not including the variables with VIF values higher than 5 (Zuur et al., 2010). Human population density was log transformed, the land use variables were log ratio transformed to normalize their distributions (Aitchison, 1986). In addition, the urbanization index was re-scaled to avoid any negative values. Tukey's test was used for Post-hoc comparisons. The best final model selection was based on Akaike's Information Criterion (AIC), and parameters were estimated by model averaging of all models with AIC ≤ 2, which were considered to be similarly supported by the data (Symonds and Moussalli, 2011).

Two sets of analyses were performed. First, we fitted a general model including body mass as the dependent variable. Date and hour of capture, wing chord and urbanization indexes (both linear and quadratic terms) were used as independent continuous variables and bird age, bird sex, bird infection status by *Haemoproteus*, *Plasmodium* and *Leucocytozoon* and habitat (urban/rural/natural) as categorical factors. Locality nested by triplet and triplet nested by province were included as random factors in the analyses (Appendix A, Table A.1.).

Second, due to the differences found in the bird body condition with respect to the urbanization index (see section 3.2.), further analyses were conducted separately for birds from each habitat category (urban/rural/natural). Thus, three models were fitted including body mass as the dependent variable. Date and hour of capture, wing chord, percentage of area covered by urban, crop and natural lands as well as human density were incorporated as independent continuous variables; and bird age, bird sex, bird infection status by *Haemoproteus*, *Plasmodium* and *Leucocytozoon* were included as independent categorical factors. Locality nested by province were considered as a random factor (Appendix A, Table A.2.).

Due to the significance of bird age (see results), each analysis was carried out separately including: 1) both adults and yearlings, 2) only adults or 3) only yearlings. The variance explained by these models was calculated using both the marginal R^2 (proportion of variance explained only by the fixed factors) and the conditional R^2 (proportion of variance considering both fixed and random factors) following Nakagawa and Schielzeth (2013). Finally, variances in body mass from urban birds of all ages were compared between infected and not infected individuals using Bartlett test (Crawley, 2007).

Results

Prevalence of blood parasites

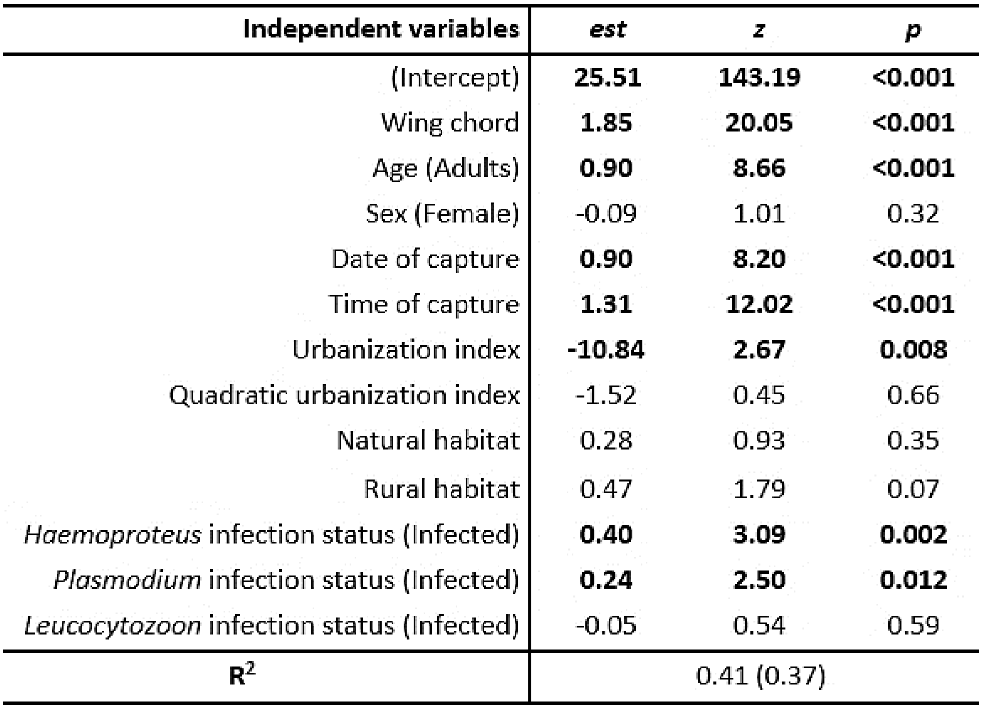
Overall, 59% of the 2,043 individuals analysed for this study were infected by at least one parasite genus. The prevalence of *Plasmodium* (29%) and *Leucocytozoon* (30%) parasites were similar and higher than the prevalence of *Haemoproteus* (16%). We found a mean prevalence of infection of 52%, 60% and 67% in urban, rural and natural habitats, respectively. The highest prevalence of infection by *Plasmodium* parasites was found in rural habitats (33%), followed by natural (30%) and urban (25%) habitats. For the case of *Leucocytozoon* parasites, the highest prevalence was found in natural habitats (36%) followed by urban (31%) and rural habitats (25%). Finally, *Haemoproteus* parasites were more prevalent in natural habitats (22%), followed by rural (16%) and urban habitats (12%). However, the prevalence of the three blood parasite genera did not differ statistically between habitat categories (data not shown). See further information in Appendix B, Table B.1 and Appendix C, Table C.1.

Bird body condition and habitat categories

Older birds showed a higher body condition (Table 1). Considering all age categories, bird body condition was negatively associated with the urbanization index and tended but not significantly to be higher in rural habitats compared with urban ones (Table 1). However, both the urbanization index variable and the habitat categories reflect very similar information, the first one as a continuous variable and the second one as categories. Consequently, when urbanization index (and its quadratic) are removed from the model, the statistical significance of habitat increases (χ^2=7.45, d.f.=2, *p*=0.02) and post-hoc test confirms that birds from rural habitats had a higher body condition than those from urban habitats (Rural–Urban: est=0.58, z=2.84, *p*=0.01), while body condition did not differ between birds from rural and natural habitats (Rural–Natural: est=0.16, z=0.77, *p*=0.72) and between birds from natural and urban habitats (Natural–Urban:

est=0.42, z=1.95, *p*=0.13). Bird body condition was positively related with the prevalence of infection by *Plasmodium* or *Haemoproteus* parasites after controlling for the significant effect of wing chord, bird age, date and time of capture (Table 1).

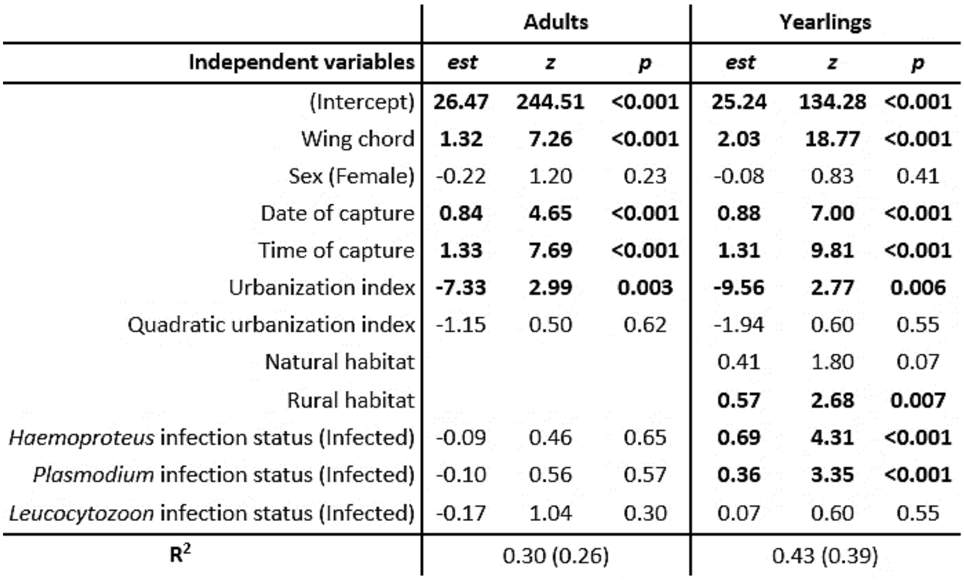
Table 1. Results of the LMMs on the effect of urbanization on the bird body condition including the three habitat categories and all ages (N=2,043). Body mass was included in the analysis as a dependent variable. Estimate (*est*), *z* and *p* values from the models are shown. Significant associations ($p<0.05$) are shown in bold. Conditional and marginal (in brackets) R^2 values are shown.



Independent variables	***est***	***z***	***p***
(Intercept)	**25.51**	**143.19**	**<0.001**
Wing chord	**1.85**	**20.05**	**<0.001**
Age (Adults)	**0.90**	**8.66**	**<0.001**
Sex (Female)	-0.09	1.01	0.32
Date of capture	**0.90**	**8.20**	**<0.001**
Time of capture	**1.31**	**12.02**	**<0.001**
Urbanization index	**-10.84**	**2.67**	**0.008**
Quadratic urbanization index	-1.52	0.45	0.66
Natural habitat	0.28	0.93	0.35
Rural habitat	0.47	1.79	0.07
Haemoproteus infection status (Infected)	**0.40**	**3.09**	**0.002**
Plasmodium infection status (Infected)	**0.24**	**2.50**	**0.012**
Leucocytozoon infection status (Infected)	-0.05	0.54	0.59
R^2	0.41 (0.37)		

The body condition of yearlings was negatively related to urbanization and was significantly higher in rural habitats compared with urban ones. Also tended, but not significantly, to be higher in natural compared with urban habitats. Yearling birds body condition was likewise higher for individuals infected by *Plasmodium* or *Haemoproteus* after controlling for the significant effect of wing chord, date and time of capture (Table 2). Body condition of adult birds was negatively associated to the urbanization index, while no relationship was found in relation to haemosporidians infection after controlling for the significant effect of wing chord, date and time of capture (Table 2).

Table 2. Results of the LMMs on the effect of urbanization on the bird body condition including the three habitat categories in adults (N=542) and yearlings (N=1,501). Body mass was included in the analysis as a dependent variable. Only estimate (*est*), *z* and *p* values from the independent variables included in the final models are shown. Significant associations ($p<0.05$) are shown in bold. Conditional and marginal (in brackets) R^2 values are shown.



	Adults			**Yearlings**		
Independent variables	***est***	***z***	***p***	***est***	***z***	***p***
(Intercept)	**26.47**	**244.51**	**<0.001**	**25.24**	**134.28**	**<0.001**
Wing chord	**1.32**	**7.26**	**<0.001**	**2.03**	**18.77**	**<0.001**
Sex (Female)	-0.22	1.20	0.23	-0.08	0.83	0.41
Date of capture	**0.84**	**4.65**	**<0.001**	**0.88**	**7.00**	**<0.001**
Time of capture	**1.33**	**7.69**	**<0.001**	**1.31**	**9.81**	**<0.001**
Urbanization index	**-7.33**	**2.99**	**0.003**	**-9.56**	**2.77**	**0.006**
Quadratic urbanization index	-1.15	0.50	0.62	-1.94	0.60	0.55
Natural habitat				0.41	1.80	0.07
Rural habitat				**0.57**	**2.68**	**0.007**
Haemoproteus infection status (Infected)	-0.09	0.46	0.65	**0.69**	**4.31**	**<0.001**
Plasmodium infection status (Infected)	-0.10	0.56	0.57	**0.36**	**3.35**	**<0.001**
Leucocytozoon infection status (Infected)	-0.17	1.04	0.30	0.07	0.60	0.55
R^2	0.30 (0.26)			0.43 (0.39)		

Independent models considering the local landscape characteristics were fitted for each of the three habitats. In urban habitats bird body condition decreased with the area covered by urbanized lands and in rural habitats it appears as a tendency (Table 3). Significant associations between bird body condition and parasite infection status were only found in birds from urban habitats where *Plasmodium* and *Haemoproteus* infected birds showed a higher body condition than uninfected ones (Table 3). Body condition of birds from rural and natural areas was not related significantly to any of the land cover or infection status variables (Table 3).

Table 3. Results of the LMMs on the effect of habitat uses and infection status on the bird body condition in the three habitat categories: urban (N=735), rural (N=717) and natural (N=591). Body mass was included in the analyses as the dependent variable. Only estimate (*est*), *z* and *p* values from the independent variables included in the final models are shown. Significant associations ($p<0.05$) are shown in bold. Conditional and marginal (in brackets) R^2 values of the models are shown. Conditional and marginal (in brackets) R^2 values are shown.

	Urban			Rural			Natural		
Independent variables	***est***	***z***	***p***	***est***	***z***	***p***	***est***	***z***	***p***
(Intercept)	**25.08**	**202.37**	**<0.001**	**26.05**	**237.88**	**<0.001**	**25.62**	**179.63**	**<0.001**
Wing chord	**2.26**	**14.32**	**<0.001**	**1.50**	**9.23**	**<0.001**	**1.69**	**11.03**	**<0.001**
Age (Adults)	**0.90**	**5.19**	**<0.001**	**1.02**	**6.02**	**<0.001**	**0.55**	**2.75**	**0.006**
Sex (Female)				-0.25	1.65	0.10			
Date of capture	**0.83**	**4.56**	**<0.001**	**0.96**	**5.42**	**<0.001**	**0.98**	**4.98**	**<0.001**
Time of capture	**1.26**	**7.30**	**<0.001**	**1.09**	**6.10**	**<0.001**	**1.40**	**8.36**	**<0.001**
Crops land coverage	0.29	1.15	0.25	-0.10	0.36	0.72	-0.13	0.47	0.64
Urbanized land coverage	**-0.68**	**2.58**	**0.010**	-0.41	1.85	0.06	-0.11	0.42	0.68
Natural land coverage	-0.24	0.95	0.34				-0.09	0.30	0.76
Population density				-0.11	0.51	0.61	-0.13	0.67	0.50
Haemoproteus infection status (Infected)	**0.82**	**3.40**	**<0.001**				0.27	1.46	0.14
Plasmodium infection status (Infected)	**0.52**	**3.02**	**<0.001**	0.11	0.76	0.45			
Leucocytozoon infection status (Infected)				-0.25	1.46	0.14	0.30	1.85	0.06
R^2	0.46 (0.44)			0.35 (0.33)			0.40 (0.35)		

Bird body condition decreased with the area covered by urbanized lands and increase with the infection of *Haemoproteus* or *Plasmodium* in yearling birds from urban habitats (Table 4). Similarly, in urban adult birds, a negative association was found between the area covered by urbanized land and bird body condition, but body condition was unrelated to the infection by any haemosporidian (Table 4). Body condition of adults and yearlings from rural and natural habitats was not related to land cover variables nor haemosporidian infection status (Appendix D, table D.1. and table D.2.).

Finally, although *Plasmodium* and *Haemoproteus* infected birds from urban habitats showed a higher body condition, birds infected by *Plasmodium* had a lower variance in body mass than uninfected individuals (Fig. 2; Bartlett test, *Plasmodium*: all ages, K^2=7.51, p=0.006; *Haemoproteus*: all ages, K^2=2.93, p=0.09). This lower variance was confirmed when analysed yearling birds (*Plasmodium*, K^2=4.92, p=0.026; *Haemoproteus*, K^2=1.02, p=0.31) but not for adult birds (*Plasmodium*, K^2=0.05, p=0.82; *Haemoproteus*, K^2=0.14, p=0.71).

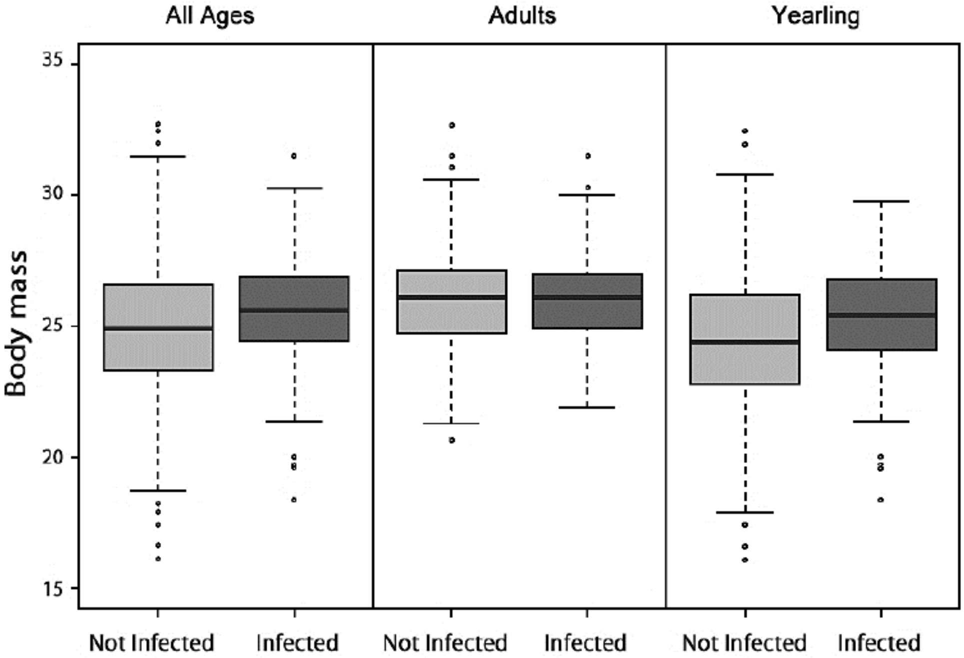


Figure 2. Boxplots of bird body mass in urban areas with respect to the infection status by *Plasmodium*: infected (black) and not infected (grey) birds according to the age categories (yearlings, adults or both). Boxplots show median values, the lower (Q1) and upper (Q3) quartile and the range (9-91%).

Table 4. Results of the LMMs on the effect of habitat uses and infection status on the bird body condition in urban areas. Body mass was included in the analyses as the dependent variable. Overall, data from adults (N=225) and yearlings (N=510) was analysed. Only estimate (*est*), *z* and *p* values from the independent variables included in the final models are shown. Significant associations ($p<0.05$) are shown in bold. Conditional and marginal (in brackets) R^2 values of the models are shown.

	Adults			**Yearlings**		
Independent variables	***est***	***z***	***p***	***est***	***z***	***p***
(Intercept)	**26.06**	**225.59**	**<0.001**	**24.68**	**152.28**	**<0.001**
Wing chord	**1.06**	**3.67**	**<0.001**	**2.68**	**13.74**	**<0.001**
Sex (Female)	**-0.54**	**1.98**	**0.048**	0.10	0.57	0.57
Date of capture	0.33	1.35	0.18	**0.92**	**4.26**	**<0.001**
Time of capture	**1.14**	**4.73**	**<0.001**	**1.17**	**5.32**	**<0.001**
Crops land coverage	0.26	1.08	0.28	0.19	0.56	0.57
Urbanized land coverage	**-0.76**	**3.13**	**0.002**	**-0.77**	**2.14**	**0.032**
Natural land coverage				-0.15	0.47	0.64
Population density				0.16	0.53	0.60
Haemoproteus infection status (Infected)	-0.18	0.57	0.57	**1.29**	**3.97**	**<0.001**
Plasmodium infection status (Infected)	0.21	0.82	0.41	**0. 68**	**3.21**	**0.001**
Leucocytozoon infection status (Infected)	-0.14	0.57	0.57	0.07	0.36	0.72
R^2	0.25 (0.25)			0.52 (0.49)		

Discussion

Results from this study provide strong evidence supporting the impact of human land-use transformation on the body condition of wild birds. Although the use of bird body mass as a measure of bird body condition has been debated, this variable is easy to measure, repeatable and allow to describe energy and nutrient reserves (Labocha and Hayes, 2012). Different methods have been proposed to control for or remove the effects of individual size on body mass (i.e. Bókony et al., 2012; Peig and Green, 2009), and in this study we have done so by including individual size (wing chord) as a covariate on the analyses with body mass as dependent variable as recommended by Garcia-Berthou (2001). After controlling for the effect of wing chord and other variables potentially affecting body mass (i.e. age, sex, date and hour of capture) we obtained a simplistic body condition index as previously used in studies on the effect of urbanization on bird populations (Liker et al., 2008). Using this approach, we found that birds from urban habitats had lower body condition than rural birds. Additionally, extend of urbanization was negatively related to bird body condition in urban habitats. However, it was surprising that yearlings infected by *Plasmodium* or *Haemoproteus* parasites in urban areas had a higher body condition than uninfected individuals.

Effect of landscape on bird body condition

When comparing birds from habitats with different characteristics, urbanization score was negatively related to the body condition of house sparrows. In the urban habitats, the surface covered by urbanized areas was negatively linked with bird body condition. Similar conclusions were also found by Bókony et al. (2012) and Liker et al. (2008) who reported a negative association between urbanization and the body mass of house sparrows (but see Meillère et al. (2016)). Different reasons may explain these results. Urban stressors like chemical pollutants affect bird health (Isaksson, 2015) potentially decreasing the body condition of both adult (Dulisz et al., 2016; but see Salmón et al., 2018) and nestling birds (Salmón et al., 2016), as shown in this study. Also, birds exposed to air pollution in cities may upregulate the antioxidant machinery (Herrera-Dueñas et al., 2014; Salmón et al., 2018) and trace element contamination may increase the stress response in birds (Meillère et al., 2016). Urban stressors including chemical, noise, artificial light pollution, diseases and diet may increase the oxidative stress of organisms and their responses against these stressors to maintain homeostasis (e.g. synthesis of HSPs) are energetically costly (Chávez-Zichinelli et al. 2010; Isaksson 2015), finally reducing the body condition of individuals from urban habitats.

The low concentrations of essential nutrients found in urban food resources (Heiss et al., 2009; Meyrier et al., 2017), may negatively affect the health status of wild birds (Dulisz et al., 2016). Thus, both urban pollution and dietary differences could explain our results (see Salmón et al. (2016)). However, urban habitats offer more abundant and seasonally stable food sources (Shochat et al., 2006). Under this scenario, birds do not need to accumulate energy reserves in their bodies (Salleh Hudin et al., 2016). Thus, we cannot rule out the alternative that birds from urban habitats had a lower body condition because of a higher food predictability in these habitats. Nevertheless, food is as well predictable in rural habitats where birds may feed on food resources provided to farm animals and we found that birds from rural habitats had higher body condition than those from urban habitats, especially during the earliest stages of life. Liker et al., (2008) found similar differences between urban and rural bird body mass that remained during an experiment with ad libitum access to food, supporting our results. These authors suggested that these differences in body condition could originate during nestling development.

The availability of high-quality food for house sparrows (e.g. food storage or feeders used by livestock) in rural habitats can explain the higher body condition of birds from this habitat (Salleh Hudin et al., 2016). Historically, the expansion of house sparrows was favoured by an increased amount of food available at human agricultural settlements (SÆtre et al., 2012). Cropland species, such as house sparrows, reach higher richness in borders surrounding natural areas and are more dependent on the abundance of crops and on landscape diversity (Pino et al., 2000).

Effects of parasitism on bird body condition

Host-parasite interactions may vary with habitat urbanization (Delgado-V. and French, 2012). Environmental factors that affect bird community abundance and composition, may also affect vector development and abundance (Ferraguti et al., 2016), which in last term may determine the differences found between habitats in the prevalence and risk of infection of parasites (Lachish et al., 2013). The effects of parasite infections on vertebrate hosts may be modulated by the environmental conditions where they occur. The degree of virulence of avian Haemosporidians have been largely discussed with studies reporting positive (Bichet et al., 2013), negative (Martínez-de la Puente et al., 2010; Marzal et al., 2008, 2005; Merino et al., 2000) or even non-significant (Carrete et al., 2009) associations between parasite infection status and the body condition of wild birds.

We found a positive relationship between *Plasmodium* and *Haemoproteus* infections and bird body condition, which was only significant in yearling birds from urban habitats. These unexpected results could be explained by a differential mortality rate of infected birds with respect to uninfected ones. If only infected birds showing a better body condition were able to face and survive parasite infections (Houston et al., 2007; Moreno-Rueda, 2011; Navarro et al., 2003), it is possible that infected individuals showed a higher body condition than uninfected birds (Sorci, 2013). Moreover, the mortality cost of malaria infections occurs during a short stage of high parasitaemia, and after such period surviving individuals harbour chronic infections with lower fitness costs (Bensch et al., 2007; Valkiūnas, 2004, but see Asghar et al., 2015; Martínez-de la Puente et al., 2010). Thus, yearling birds who probably get infected right after or during nestling time when they are immunologically naive (Merino, 2010), may suffer a higher impact of parasite infections than adults. Which may explain that in this study, we found differences in body mass in relation to infection status only in yearlings.

Moreover, Fokidis et al., (2008) found that a higher energetic cost was associated to immunological responses against infections in urban birds, rather than in birds from rural environments. This costly immunity activation caused a decrease in haptoglobin production and a loss of body condition (Bailly et al., 2016). The higher expression of immunoregulatory genes found in urban habitats with respect to those from natural environments may also explain the higher body condition of infected vs uninfected birds in urban habitats (Watson et al., 2017).

Conclusions

Our results support the adverse effects of urbanization on bird body condition, although local scale habitat characteristics and the infection status by Haemosporidian parasites, may modulate these relationships. Therefore, birds in urban habitats may suffer, among other potential factors, the synergistic effects of pollution, bad quality diet, and parasite infections, especially during the earliest stages of life. Also, our results suggest that in urban habitats, only those individuals showing a higher body condition were able to survive to parasite infection while dealing with these other adverse factors.

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Ethical approval

This study was done with all the necessary permits issued by the regional Department of Environment (Consejería de Medio Ambiente, Junta de Andalucía) and all the animal experimentation licences required according to Spanish law. The CSIC Ethics Committee approved the experimental procedures on 9th March 2012. Surveys on private lands were conducted with the verbal consent of the owners. Birds were released in the area immediately after sampling without any apparent damage.

Conflict of interest

The authors declare they have no actual or potential competing financial interest.

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Supplementary material

Appendix A

Table A.1. Dependent (a), independent variables (b) and random factors (c) included in the LMMs global model.

	VARIABLE NAME	VARIABLE DESCRIPTION
a)	Body mass	Bird body mass (gr)
b)	Wing chord	Bird wing length (mm)
	Age	Bird age classified as yearling (born the same year, 3) or adult (> 1 year old, 4)
	Sex	Bird sex, male (1) or female (2)
	Date of capture	Day of capture (1st January = 1)
	Hour of capture	Hour of capture (00:00 = 0; 23:59 = 0.99)
	Urbanization index	Degree of urbanization at the sampling area estimated from google maps images
	Quadratic urbanization index	Quadratic of urbanization index
	Habitat	Habitat category (urban, rural or natural)
	Haemoproteus infection status	Infection status by *Haemoproteus* (infected (1) or uninfected (0))
	Plasmodium infection status	Infection status by *Plasmodium* (infected (1) or uninfected (0))
	Leucocytozoon infection status	Infection status by *Leucocytozoon* (infected (1) or uninfected (0))
c)	Province	Geographical province where the sample point belongs to
	Triplet	Trio of geographically close localities/sample point with different habitat categories
	Locality	Sample point

Table A.2. Dependent (a), independent variables (b) and random factors (c) included in the LMMs partial model of each habitat (urban, rural and natural).

	VARIABLE NAME	VARIABLE DESCRIPTION
a)	Body mass	Bird body mass (g)
b)	Wing chord	Bird wing length (mm)
	Age	Bird age classified as yearling (born the same year) (3) or adult (> 1 year old) (4)
	Sex	Bird sex, male (1) or female (2)
	Date of capture	Day of capture (1st January = 1)
	Hour of capture	Hour of capture (00:00 = 0; 23:59 = 0.99)
	Crops land cover	Percentage of surface with crops in a buffer of 2000 m around the sampling area
	Urbanized land cover	Percentage of urbanized surface in a buffer of 2000 m around the sampling area
	Natural land cover	Percentage of natural surface in a buffer of 2000 m around the sampling area
	Population density	Density of Human population (humans/250 m^2) in each locality
	Haemoproteus infection status	Infection status by *Haemoproteus* (infected (1) or uninfected (0))
	Plasmodium infection status	Infection status by *Plasmodium* (infected (1) or uninfected (0))
	Leucocytozoon infection status	Infection status by *Leucocytozoon* (infected (1) or uninfected (0))
c)	Province	Geographical province where the sample point belongs to
	Locality	Sampling point

Appendix B

Table B.1. Mean ± S.E. for the independent and dependent variables analysed in this study.

Province	**Triplet**	**Habitat**	**Urbanization index**	**Sample size**	**Wing chord average**	**Body mass average**	***Haemoproteus* prevalence (%)**	***Plasmodium* prevalence (%)**	***Leucocytozoon* prevalence (%)**	**Haemosporidian prevalence (%)**
Cadiz	Chipiona	Urban	4.020	46	75.83	26.81	0.00	26.09	32.61	47.83
		Rural	1.653	23	77.08	26.97	4.35	26.09	56.52	60.87
		Natural	0.035	14	77.39	26.18	0.00	64.29	28.57	78.57
	Jerez	Urban	3.068	4	73.87	27.97	0.00	25.00	50.00	75.00
		Rural	-1.986	33	77.54	27.67	6.06	30.30	24.24	54.55
		Natural	-1.821	51	76.31	26.11	9.80	50.98	31.37	70.59
	Puerto de Santa Maria	Urban	2.039	11	74.13	24.63	0.00	9.09	9.09	18.18
		Rural	1.643	50	75.82	27.36	6.00	10.00	4.00	20.00
		Natural	-0.170	4	72.87	27.47	0.00	0.00	0.00	0.00
	Rota	Urban	0.701	22	77.14	27.92	4.55	31.82	50.00	63.64
		Rural	-2.070	41	75.11	25.60	4.88	17.07	26.83	48.78
		Natural	-0.921	28	78.32	26.05	3.57	17.86	10.71	25.00
	Sanlucar Barrameda	Urban	5.224	16	75.69	26.52	6.25	18.75	0.00	25.00
		Rural	1.013	46	75.37	26.16	0.00	36.96	0.00	36.96
		Natural	-1.394	5	78.20	28.36	20.00	60.00	40.00	80.00

Table B.1.(cont.) U.I. : Urbanization index ; S.S. : Sample size ; W.C. : Wing chord ; B.M. : Body mass ; H.P. : *Haemoproteus* prevalence (%) ; P.P. : *Plasmodium* prevalence (%) ; L.P. : *Leucocytozoon* prevalence (%) ; H.P. : Haemosporidian prevalence (%).

Province	Triplet	Habitat	U.I.	S.S.	W.C.	B.M.	H.P.	P.P.	L.P.	H.P.
Huelva	Beas	Urban	-0.200	77	72.52	25.62	49.35	20.78	42.86	80.52
		Rural	-0.611	81	73.03	25.74	80.25	12.35	41.98	92.59
		Natural	-1.234	66	74.49	26.41	69.70	16.67	53.03	89.39
	Doñana	Urban	1.172	91	72.91	25.16	2.20	46.15	37.36	70.33
		Rural	-1.142	85	73.39	25.88	3.53	55.29	2.35	60.00
		Natural	-1.603	9	76.11	26.37	22.22	55.56	0.00	77.78
	Gibraleón	Urban	1.922	95	72.35	25.06	26.32	25.26	78.95	85.26
		Rural	-1.777	58	73.97	26.41	13.79	25.86	68.97	75.86
		Natural	-1.234	62	73.15	25.38	22.58	20.97	85.48	88.71
	Huelva	Urban	3.898	89	72.11	23.10	2.25	11.24	6.74	16.85
		Rural	-1.973	67	73.35	25.74	2.99	38.81	4.48	43.28
		Natural	-0.574	52	74.13	26.07	1.92	32.69	7.69	40.38
	San Juan del Puerto	Urban	0.799	91	73.07	25.68	14.29	48.35	14.29	67.03
		Rural	-2.159	72	73.95	27.35	20.83	37.50	20.83	65.28
		Natural	-2.912	63	74.41	26.79	39.68	42.86	36.51	87.30

Table B.1.(cont.) U.I. : Urbanization index ; S.S. : Sample size ; W.C. : Wing chord ; B.M. : Body mass ; H.P. : *Haemoproteus* prevalence (%) ; P.P. : *Plasmodium* prevalence (%) ; L.P. : *Leucocytozoon* prevalence (%) ; H.P. : Haemosporidian prevalence (%).

Province	**Triplet**	**Habitat**	**U.I.**	**S.S.**	**W.C.**	**B.M.**	**H.P.**	**P.P.**	**L.P.**	**H.P.**
Sevilla	Cañada	Urban	0.516	69	74.88	24.08	1.45	4.35	11.59	17.39
		Rural	-2.130	23	75.87	25.81	21.74	56.52	13.04	78.26
		Natural	-1.051	93	74.55	24.67	27.96	17.20	16.13	50.54
	Guadaira	Urban	2.216	18	76.83	25.59	22.22	16.67	66.67	83.33
		Rural	-2.768	20	75.62	24.71	15.00	50.00	25.00	80.00
		Natural	-2.885	63	75.76	24.73	6.35	19.05	46.03	58.73
	Lantejuela	Urban	-0.221	22	75.59	25.77	4.55	54.55	63.64	90.91
		Rural	1.874	38	74.72	24.54	5.26	31.58	60.53	73.68
		Natural	-2.235	49	75.59	25.77	6.12	40.82	44.90	71.43
	Sevilla	Urban	5.214	4	74.62	24.85	0.00	0.00	25.00	25.00
		Rural	-0.888	33	74.20	24.26	3.03	27.27	45.45	63.64
		Natural	-0.411	9	75.17	24.81	11.11	11.11	44.44	66.67
	Veta la Palma	Urban	1.552	80	74.08	24.20	0.00	6.25	1.25	7.50
		Rural	-0.778	47	75.05	25.62	4.26	40.43	8.51	48.94
		Natural	-1.415	23	74.24	24.19	4.35	60.87	0.00	65.22

Appendix C

Table C.1. Prevalence (%) of infection by each blood parasite genera (*Plasmodium, Haemoproteus* and *Leucocytozoon*) and infection status (Haemosporidians) per habitat (urban, rural and natural) and category age (3: yearling; 4: adult > 1 year old; All: all ages).

	Plasmodium			***Haemoproteus***		
	3	**4**	**All**	**3**	**4**	**All**
Urban	23.14	28.89	24.90	8.63	19.56	11.97
Rural	36.69	20.00	32.50	11.55	28.89	15.90
Natural	31.50	26.28	30.29	18.06	35.04	22.00
Total	30.51	25.28	29.12	12.52	26.57	16.25

	Leucocytozoon			**Haemosporidians**		
	3	**4**	**All**	**3**	**4**	**All**
Urban	24.12	45.78	30.75	45.88	65.78	51.97
Rural	17.50	46.67	24.83	57.91	66.67	60.11
Natural	29.07	56.93	35.53	62.56	81.02	66.84
Total	23.25	48.89	30.05	55.23	69.93	59.13

Appendix D

Table D.1. Results of the LMMs on the effect of urbanization on bird body condition in rural habitats for adults (N=180) and yearlings (N=537). Body mass was included in the analysis as a dependent variable. Estimate (*est*), *z* and *p* values from the models are shown. Significant associations ($p<0.05$) are shown in bold. Conditional and marginal (in brackets) R^2 values of the models are shown.

	Adults			**Yearlings**		
Independent variables	***est***	***z***	***p***	***est***	***z***	***p***
(Intercept)	**27.05**	**184.70**	**<0.001**	**25.74**	**193.51**	**<0.001**
Wing chord	**1.54**	**5.32**	**<0.001**	**1.51**	**8.71**	**<0.001**
Sex (Female)				-0.26	1.59	0.11
Date of capture	**1.44**	**4.68**	**<0.001**	**0.85**	**4.11**	**<0.001**
Time of capture	**1.48**	**4.86**	**<0.001**	**0.92**	**4.02**	**<0.001**
Crops land coverage				-0.25	0.70	0.48
Urbanized land coverage	-0.33	0.99	0.32	-0.50	1.83	0.07
Population density	-0.28	0.87	0.38	-0.20	0.72	0.47
Haemoproteus infection status (infected)	-0.51	1.56	0.12	0.36	1.28	0.20
Plasmodium infection status (infected)	-0.26	0.71	0.48	0.19	1.13	0.26
Leucocytozoon infection status (infected)	-0.26	0.88	0.38	-0.13	0.64	0.53
R^2	0.30 (0.30)			0.32 (0.28)		

Table D.2. Results of the LMMs on the effect of urbanization on bird body condition in natural habitats for adults (N=137) and yearlings (N=454). Body mass was included in the analysis as a dependent variable. Estimate (*est*), *z* and *p* values from the models are shown. Significant associations (p<0.05) are shown in bold. Conditional and marginal (in brackets) R^2 values of the models are shown.

	Adults			Yearlings		
Independent variables	***est***	***z***	***p***	***est***	***z***	***p***
(Intercept)	**26.30**	**143.47**	**<0.001**	**25.45**	**183.01**	**<0.001**
Wing chord	**1.41**	**4.99**	**<0.001**	**1.78**	**9.66**	**<0.001**
Sex (Female)				-0.11	0.60	0.55
Date of capture	0.37	1.25	0.21	**0.95**	**4.44**	**<0.001**
Time of capture	**1.47**	**4.75**	**<0.001**	**1.37**	**7.49**	**<0.001**
Crops land coverage				-0.16	0.60	0.56
Urbanized land coverage	0.65	1.89	0.06	-0.22	0.78	0.43
Natural land coverage	-0.18	0.52	0.60	0.11	0.44	0.66
Population density	-0.86	1.98	0.05	-0.09	0.43	0.67
Haemoproteus infection status (infected)	0.44	1.35	0.18	0.43	1.80	0.07
Plasmodium infection status (infected)	-0.46	1.48	0.14	0.27	1.44	0.15
Leucocytozoon infection status (infected)				0.36	1.93	0.05
R^2	0.32 (0.31)			0.40 (0.37)		

CHAPTER 3

DIFFERENCES IN FATTY ACIDS COMPOSITION BETWEEN *PLASMODIUM* INFECTED AND UNINFECTED HOUSE SPARROWS ALONG AN URBANIZATION GRADIENT

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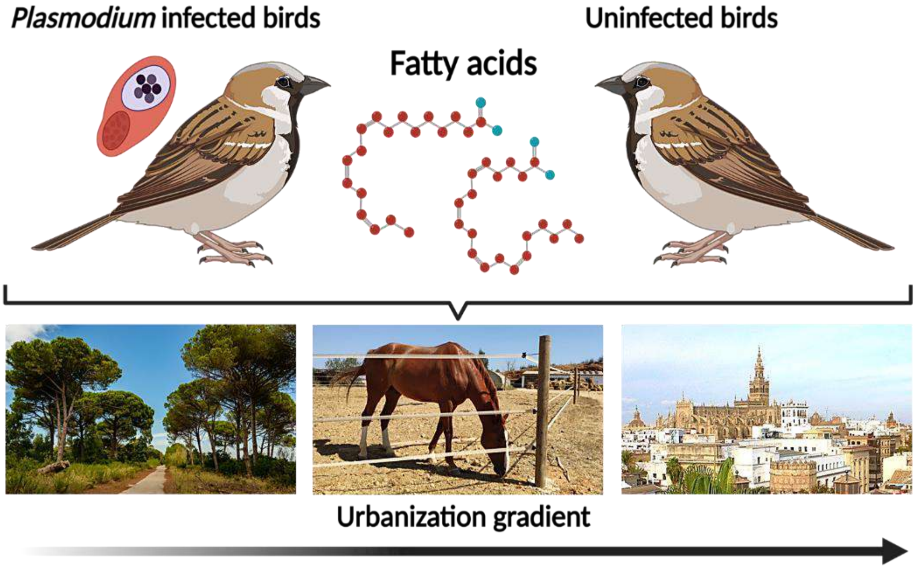
Graphical abstract



Highlights:

- Fatty acids were analyzed in birds from habitats along an urbanization gradient.
- ω-3 and ω-6 proportion differed between *Plasmodium* infected and uninfected birds.
- The ω-6/ω-3 ratio was lower in *Plasmodium* infected birds from natural habitats.
- Fatty acids composition in natural habitats may help birds to fight off infections.
- The higher concentration of fatty acids in urban birds suggests a fat-rich diet.

Keywords: Avian malaria; immune responses; *Passer domesticus*; PUFA; Omega-6; Omega-3.

Abstract

Anthropogenic activities such as intensification of agriculture, animal husbandry and expansion of cities, can negatively impact wildlife through the availability of high-quality food resources and the influence of pathogen transmission. The house sparrow (*Passer domesticus*), a common urban exploiter species, is undergoing a consistent population decline. Nutritional constrains and infectious diseases, like avian malaria, has been highlighted as potential causes. Specific fatty acids (FA) play important roles in physiological processes such as immune responses and are highly influenced by dietary availability. Previous studies show pronounced differences in the composition of FA between urban and rural birds. Similarly, avian malaria epidemiology is affected by urbanization. However, little attention has been given to the relationship between prevalence of parasites and host FA composition along landscapes with different degree of anthropization. Here, we analysed 165 juvenile house sparrows either infected by *Plasmodium* or uninfected, captured at 15 localities along an urbanization gradient. Interestingly, *Plasmodium* infected birds showed higher relative levels of ω-3 PUFAs but lower of ω-6 PUFAs than uninfected birds. In concordance, the ω-6/ω-3 ratio was also lower in infected than in uninfected birds, but only from natural habitats. These results imply that infected birds, especially from natural habitats, have a potentially beneficial composition of PUFAs for mounting an anti-inflammatory immune response. The total absolute concentration of circulating FAs was higher in urban birds than in birds from rural habitats, suggesting that in more anthropized habitats, birds have a greater availability of fat-rich foods sources. Overall, these results highlight the importance of studying bird’s physiological condition alongside with nutritional status to understand population tolerance towards parasitic infections in landscapes of varying degree of urbanization. Future studies should establish the causal link between avian malaria prevalence and FA composition to understand whether infection influences the FA composition and food preference or vice versa.

Introduction

The intensification of agriculture, animal husbandry and the expansion of cities, has changed the environmental conditions around the globe (Bradley and Altizer, 2007; Grimm et al., 2000). These human activities can reduce local biodiversity and alter interactions between organisms, such as disease transmission (Bradley and Altizer, 2007; Seress and Liker, 2015; Sol et al., 2014). However, some species are able to thrive with those anthropogenic environments. Cities can provide favourable conditions for birds such as milder climate during winter (Saaroni et al., 2000), higher availability of nesting sites (Sumasgutner et al., 2014), reduced density of predators (Møller and Díaz, 2017; but see Dulisz et al., 2016) and higher predictability of food and water (Fokidis et al., 2008). Nevertheless, urban-dwelling individuals seem to pay a cost in terms of, among others, reduced body size and condition, higher oxidative stress, lower reproductive success and accelerated telomere attrition (Chamberlain et al., 2009; Jiménez-Peñuela et al., 2019; Liker et al., 2008; Salmón et al., 2016; Sepp et al., 2018; Sumasgutner et al., 2014; Watson et al., 2017). The house sparrow (*Passer domesticus*) is a sedentary urban exploiter species, that is historically known to thrive in areas with human activities and settlements, taking advantage of food resources and cavities for nesting (Hanson et al., 2020; Mckinney, 2002). However, this species is undergoing a consistent population decline across the whole of Europe, especially in cities (Anderson, 2007; Hanson et al., 2020; Laet and Summers-Smith, 2007). Nutritional constrains, intensive animal husbandry and agriculture, pollution, pesticides and infectious diseases caused by blood parasites, have been suggested as potential causes for this population decline (Bichet et al., 2013; Dadam et al., 2019; Herrera-Dueñas et al., 2017, 2014; Meillère et al., 2017; Meyrier et al., 2017; Peach et al., 2018; von Post et al., 2012).

Food with an anthropogenic origin (i.e., human waste), is usually rich in carbohydrates and fats but poor in protein content. If consumed regularly, this can have negative consequences for the fitness of wild birds (Heiss et al., 2009;

Mckinney, 2002; Meyrier et al., 2017). For Passeriformes, invertebrates predominate the diet of chicks, but the lack of suitable prey in anthropized environments and the consequential substitution by human food may strongly influence fledgling growth and survival, and thereby, nesting success and population demography (Anderson, 2007; Peach et al., 2018; Salleh Hudin et al., 2016). Good quality food allows chicks to grow faster, obtain a higher body condition and a better immunocompetence (Peach et al., 2018; Seress et al., 2019; Twining et al., 2016b). Nutrient rich food is especially important during early life stages to fulfil physiological requirements during development (Chamberlain et al., 2009; Seress et al., 2019; Twining et al., 2018). The food type consumed influence the intake of different key substances like fatty acids (FA) and thereby, their composition in these organisms, especially in the circulatory system (Finke et al., 2020).

FA are physiological components that play an important role in biological structures, like phospholipids in cell membranes, and in processes such as oxidative stress and immune responses (Arnold et al., 2015; Hulbert et al., 2005; Isaksson et al., 2017). There are three major groups of FAs: (1) saturated fatty acids (SFAs), (2) monounsaturated fatty acids (MUFAs) and (3) polyunsaturated fatty acids (PUFAs). These are divided into omega-6 (ω-6) and omega-3 (ω-3) PUFAs, with different biological functions each (Hulbert et al., 2005). Recent studies have shown differences in plasma and yolk FA composition between urban and rural birds, but most importantly differences are found in the ω-6 and ω-3 PUFAs (Andersson et al., 2015; Isaksson et al., 2017; Toledo et al., 2016). These long-chain PUFAs can modulate both cellular and humoral immune responses by affecting the production of lymphocytes, heterophils and splenocytes as well as IgM and IgG antibodies (Alagawany et al., 2019). Specifically, ω-6 PUFAs are metabolized to pro-inflammatory prostaglandins, while the ω-3 PUFAs are metabolized into anti-inflammatory agents, so an alteration in the ω-6/ω-3 ratio has been associated with changes in immune responses and in oxidative stress (Arnold et al., 2015; Chang et al., 2018; Hulbert et al., 2005; Simopoulos, 2011). Indeed, under higher exposure to pro-oxidants caused by internal inflammatory responses or external substances (i.e. urban pollution), unsaturated fatty acids are prone to oxidation (lipid peroxidation) which affects their structure and thereby their function (Isaksson et al., 2017; Pamplona et al., 2002, 2000).

Immune responses are energetically costly, so the capacity of the host to effectively fight off infections are determined by their current body condition and nutritional status (Cornet et al., 2014; Delhaye et al., 2018; Navarro et al., 2003). Avian malaria of the genus *Plasmodium* are common blood parasites (Atkinson et al., 1991; Valkiūnas, 2005) with detrimental effects on health and survival of birds (Martínez-de la Puente et al., 2010; Marzal et al., 2005; Merino et al., 2000). The infectious dynamics and epidemiology of these parasites has been altered in anthropized landscapes (Bichet et al., 2013; Bradley and Altizer, 2007; Calegaro-Marques and Amato, 2014; Ferraguti et al., 2018; Geue and Partecke, 2008). Despite the well-known implications of avian malaria and the importance of a low ω-6/ω-3 ratio for mounting an anti-inflammatory immune response, there is no study to date that investigate how these two factors are related in birds living in environments with different degree of anthropization. Here, we investigate the FA composition of juvenile house sparrows either infected by *Plasmodium* or uninfected along an urbanization gradient including urban, rural, and natural habitats. We measured the relative proportion of the three main FA groups SFA, MUFA and PUFA, along with the two PUFA groups ω-3 and ω-6, and the absolute total FA concentration (FA_{tot}). In addition, we also included in our analyses two biomarkers, namely the ω-6/ω-3 ratio and the Peroxidation Index (PI), a proxy for susceptibility of lipids to peroxidation (i.e., susceptibility for oxidative stress).

Materials and Methods

Study area and bird sampling

Juvenile house sparrows were caught using mist-nets and a bird call playback recorder at 15 localities of southwest Spain from July to October of 2013 (Fig. S1).

Localities were grouped in 5 triplets, each triplet included one urban, one rural and one natural habitat. Urban habitats correspond to highly populated areas, rural habitats were selected based on the presence of a high density of livestock and natural habitats were characterized as higher preserved habitats with a comparatively lower density of both livestock and humans (for further information about habitat characterization, see Ferraguti et al., 2016).

Each bird was ringed with an individual numbered metal ring, and sexed and aged based on plumage characteristics and skull ossification when possible (Svensson, 2009). Bird body mass was measured with an electronic balance (to the nearest 0.1g). A blood sample was extracted from the jugular vein using a sterile syringe and never exceeding 1% of the bird´s body mass. Blood samples were stored in Eppendorf tubes maintained in cold-boxes during field work, and subsequently kept for less than 24 hours at 4ºC until centrifugation for 10 min at 1700g (4000 rpm) to separate serum and cellular fractions. The cellular fractions were frozen at -20°C and serum at -80°C until further molecular and biochemical analysis. Birds were released at the place of capture without any apparent damage.

Molecular analyses

Genomic DNA was extracted using the Maxwell®16 LEV System Research (Promega, Madison, WI, USA). Sex-determination was performed by plumage characteristics when possible, otherwise it was determined using the primer pair CHD-P2 (5’ TCTGCATCGCTAAATCCTTT3’) and CHD-P8 (5’ CTCCCAAGGATGAGRAAYTG 3’) following the protocols by Griffiths et al. (1998, 1996). The infection status and parasite identity were determined through the amplification of a 478-bp fragment of the *Plasmodium* mitochondria cytochrome *b* gene (Hellgren et al., 2004). Negative samples were analysed twice to avoid false negatives (McClintock et al., 2010). Both negative controls for the PCR reactions (at least one per plate) and DNA extractions (one per 15 samples) were included in the analysis. Positive amplifications were sequenced unidirectionally to reduce the cost of sequencing a large number of amplicons (Dubiec et al., 2016) by Macrogen Inc. facilities (Amsterdam, The Netherlands). Sequences were edited using the software Sequencher™ v 4.9 (Gene Codes Corp. © 1991–2009, Ann Arbor, MI 48108, USA). The parasites genera were identified by comparison with sequences deposited in GenBank (National Center for Biotechnology Information) and MalAvi databases (Bensch et al., 2009). The *Plasmodium* infection status from birds studied here have already been analysed in Ferraguti et al. (2018) and Jiménez-Peñuela et al. (2019).

Fatty acid analyses

A subsample of birds evaluated in previous studies of *Plasmodium* infection were selected for further analyses of the composition of fatty acids. Selection of individuals was based on the habitat of origin, bird age and infection status by avian *Plasmodium*. Only juveniles were included in this study, as they better reflect local habitat characteristics such as food quality and availability as well as the local parasite circulation abundance and richness during the nestling and post-fledging phase (Cosgrove et al., 2008; Valkiūnas, 2005). Moreover, to test the relationship between malaria infection on FA composition, only birds infected exclusively by *Plasmodium*, or uninfected birds were included. This allows us to avoid any confounding effects produced by other blood-parasites (e.g., *Haemoproteus* and/or *Leucocytozoon*) or co-infections in the analyses. Overall, FAs were analysed in 58, 54 and 53 house sparrows from urban, rural, and natural habitats, respectively (mean=11; S.D.=1.73, per locality). Of them, 88 were males and 77 females, corresponding to 81 *Plasmodium* infected and 84 uninfected individuals.

Fatty acids from serum samples were extracted following the protocol described in Andersson et al. (2015). Briefly, 50 µl of chloroform:methanol (2:1 v/v) was added to 5 µl of defrosted serum and left for one hour at room temperature. This volume includes an internal standard methyl (Z)-10-

heptadecenoate with a known concentration (33.3 ng/ µl) that allow us to estimate the FA absolute concentration. The organic phase was then evaporated to dryness under a gentle N2 flow followed by adding of 100 µl 0.5 M KOH solution in methanol. The mix was left in the oven at 40ºC for one hour to convert the FA into Methyl Esters (FAMEs). After methanolysis, 100 µl of 0.5 M HCl in methanol followed by 300 µl of heptane were added. The mix separates into two phases, the bottom methanol phase was removed, and the FAME extract in the heptane phase was washed with 200 µl of water twice. Finally, to completely remove all the water, anhydrous sodium sulphate salt (Na2SO4) was added to the FAME extract. The supernatant was then transferred to a glass vial for analysis by Gas Chromatography/Mass Spectrometry (GC/MS). Specifically, we used an Agilent 5975 MS coupled to an Agilent 6890 GC equipped with an HP-INNOWax capillary column [30 m, 0.25 mm id, df 0.25 µm; Agilent, CA, USA]. The oven temperature was set to 80ºC for 1 min, then increased by 10ºC/min to 230°C and was held at 230ºC for 20 min. Helium was used as carrier gas at a constant flow of 1 ml/min. FAMEs present in serum were identified by comparing their mass spectra and retention times with those of synthetic standards (Supelco 37-Component FAME Mix, Sigma-Aldrich, Stockholm, Sweden), and quantified by comparing the peak area of the measurement with the peak area of the internal standard of known concentration.

Statistical analysis

The individual FAs were grouped into their chemical class (Table 1): SFA, MUFA, ω-6 PUFA, ω-3 PUFA, and all PUFA together (PUFA_{tot}= ω-6 PUFA + ω-3 PUFA + ω-9 PUFA). All groups are presented as relative proportion (%) to the total FA concentration. The FA_{tot} concentration per individual (ng/µl) was used to get an indication of the total fat intake. Moreover, we quantified two additional biomarkers including the ω-6/ω-3 ratio, and PI. The last is estimated by adding the peroxidation potential of all individual FAs (i.e., the number of double bonds multiplied by its relative abundance) (Pamplona et al., 2000, 1998). Thus, PI was calculated as PI = [(% monoenoic × 0.025) + (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 4) + (% pentaenoic × 6) + (% hexaenoic × 8)].

For the statistical analyses, the proportion of FA groups were logit-transformed (log(y/[1-y])) (Warton and Hui, 2011) and the ω-6/ω-3 ratio and the FA_{tot} were logarithmically transformed to normalize its distribution. Linear Mixed-Effects Models (LMM) fitted by maximum likelihood were used to test the effects of habitat category and *Plasmodium* infection on the plasma FA composition of wild birds. Different models were fitted for the proportion of FA of each group (SFA, MUFA, ω-6 PUFAs, ω-3 PUFAs and $PUFA_{tot}$), the ω-6/ω-3 ratio, the PI and the FA_{tot}. Habitat category (urban, rural, and natural; categorical), infection status by *Plasmodium* (infected and uninfected; categorical), and their two-way interaction, were included as independent factors. Hour of capture (continuous), day of capture (continuous), and bird body mass (continuous) were included as covariates. Locality (categorical) nested in triplet (categorical) were included as random factors to account for the geographical stratification of the sampling design (Kuznetsova et al., 2017).

Tukey's post hoc tests were used to test for differences between pairs of habitats when the factor "Habitat" was statistically significant. Contrasts of factor interactions, from the package *phia*, were used to test for differences between levels of the interaction between habitat category and *Plasmodium* infection (Martínez, 2015). The sum of squares was calculated using an ANOVA type III. The marginal (considering only fixed factors) and conditional (considering fixed and random factors) variance explained by each model was determined by a pseudo-R^2 (Nakagawa and Schielzeth, 2013) estimated with the package MuMin (Bartón, 2019). For each LMM, the collinearity between independent variables was tested by calculating the Variance Inflation Factor (VIF) (Zuur et al., 2010). Residuals from each LMM, and all dependent variables, were checked for normality by using *qq-plots*. Statistical analyses were conducted in R (v, 3.6.0 GUI 1.70 El Capitan build; R Foundation for Statistical Computing 2018) using the packages: *arm*, *car*, *cowplot*, *ggplot2*, *lattice*, *lme4*, *lmerTest*, *lsmeans*, *MASS*, *Matrix*, *multcomp*, *MuMIn*, *nlme*, *phia* and *Rcpp*.

Results

Overall, 21 different FA were identified in juvenile house sparrow's serum. Oleic acid (MUFA), palmitic acid (MUFA), stearic acid (SFA), linolenic acid (LA, ω-6 PUFA), and arachidonic acid (AA, ω-6 PUFA) accounted for the highest proportions of FA, ranged between 8 and 27% of the total fatty acid concentration (Table 1). Table 2 shows the mean proportion of serum FA for the different groups (% of total FA concentration), the FA_{tot} (ng/µl) and the two biomarkers for each habitat category and *Plasmodium* infection status.

Table 1. Relative proportion of plasma fatty acids (% of total concentration) in urban, rural and natural habitats and from Plasmodium infected and uninfected juvenile house sparrows. Table shows mean values ± Standard Error. C:Dn-x, number of carbon atoms:double bonds-position; LA: Linoleic Acid, AA: Arachidonic acid; EPA: Eicosapentanoic acid; DPA: Docosapentanoic acid; DHA: Docosahexaenoic acid.

			All		Natural		Rural		Urban	
Fatty acid name	**C:Dn-x**	**FA class**	**Infected**	**Uninfected**	**Infected**	**Uninfected**	**Infected**	**Uninfected**	**Infected**	**Uninfected**
Myristic acid	14:0	SFA	0.43 ± 0.02	0.43 ± 0.03	0.35 ± 0.04	0.28 ± 0.03	0.50 ± 0.04	0.50 ± 0.04	0.44 ± 0.05	0.48 ± 0.04
Palmitic acid	16:0		24.20 ± 2.67	24.72 ± 2.70	22.39 ± 4.23	21.23 ± 4.25	25.98 ± 5.20	26.61 ± 4.94	24.41 ± 4.61	25.82 ± 4.71
Margaric acid	17:0		0.12 ± 0.01	0.11 ± 0.01	0.13 ± 0.02	0.14 ± 0.02	0.11 ± 0.02	0.10 ± 0.02	0.12 ± 0.01	0.10 ± 0.01
Stearic acid	18:0		16.03 ± 0.37	15.88 ± 0.42	17.52 ± 0.74	18.52 ± 0.71	15.04 ± 0.57	14.67 ± 0.60	15.42 ± 0.51	14.84 ± 0.65
Arachidic acid	20:0		0.07 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.07 ± 0.02	0.07 ± 0.01	0.05 ± 0.01	0.09 ± 0.01	0.10 ± 0.02
Palmitoleic acid	16:1n-7	MUFA	1.72 ± 0.14	1.95 ± 0.16	1.46 ± 0.26	1.04 ± 0.20	1.92 ± 0.20	2.50 ± 0.27	1.79 ± 0.26	2.18 ± 0.26
Oleic acid	18:1n-9		25.39 ± 0.90	24.80 ± 0.84	22.51 ± 1.62	19.51 ± 1.58	25.39 ± 1.13	26.45 ± 1.13	28.27 ± 1.64	27.61 ± 1.25
cis-Vaccenic acid	18:1n-7		1.21 ± 0.05	1.23 ± 0.04	1.14 ± 0.10	1.09 ± 0.07	1.28 ± 0.07	1.31 ± 0.08	1.19 ± 0.07	1.26 ± 0.07
Eicosenoic acid	20:1n-7		0.11 ± 0.01	0.10 ± 0.02	0.09 ± 0.02	0.05 ± 0.02	0.11 ± 0.02	0.10 ± 0.02	0.12 ± 0.03	0.15 ± 0.03
Mead acid	20:3n-9	ω-9 PUFA	0.82 ± 0.04	0.86 ± 0.04	0.94 ± 0.08	0.98 ± 0.07	0.80 ± 0.06	0.79 ± 0.05	0.72 ± 0.05	0.84 ± 0.06
LA	18:2n-6	ω-6 PUFA	16.52 ± 0.91	16.25 ± 0.85	17.67 ± 1.65	20.21 ± 1.53	16.38 ± 1.1	13.96 ± 1.19	15.49 ± 1.82	15.18 ± 1.46
γ-Linolenic acid	18:3n-6		0.59 ± 0.10	0.50 ± 0.09	0.81 ± 0.23	0.64 ± 0.25	0.58 ± 0.19	0.41 ± 0.12	0.37 ± 0.10	0.46 ± 0.09
Eicosadineoic acid	20:2n-6		0.01 ± 0.004	0.01 ± 0.005	0.016 ± 0.008	0.005 ± 0.005	0.01 ± 0.007	0.01 ± 0.007	0.009 ± 0.006	0.02 ± 0.01
Dihomo-γ-linolenic acid	20:3n-6		0.20 ± 0.03	0.19 ± 0.02	0.18 ± 0.06	0.08 ± 0.03	0.18 ± 0.04	0.23 ± 0.04	0.24 ± 0.05	0.25 ± 0.04
AA	20:4n-6		10.08 ± 0.38	10.40 ± 0.47	11.78 ± 0.66	13.31 ± 0.79	9.32 ± 0.63	10.06 ± 0.80	9.06 ± 0.54	8.29 ± 0.56
Adrenic acid	22:4n-6		0.37 ± 0.03	0.39 ± 0.04	0.31 ± 0.04	0.40 ± 0.06	0.41 ± 0.05	0.35 ± 0.04	0.38 ± 0.04	0.42 ± 0.08
DPA	22:5n-6		0.48 ± 0.04	0.53 ± 0.05	0.43 ± 0.07	0.61 ± 0.09	0.52 ± 0.08	0.49 ± 0.08	0.49 ± 0.07	0.51 ± 0.08
α-Linolenic acid	18:3n-3	ω-3 PUFA	0.35 ± 0.05	0.35 ± 0.05	0.46 ± 0.10	0.57 ± 0.13	0.32 ± 0.08	0.22 ± 0.05	0.27 ± 0.05	0.28 ± 0.03
EPA	20:5n-3		0.30 ± 0.06	0.21 ± 0.04	0.54 ± 0.14	0.23 ± 0.09	0.19 ± 0.07	0.15 ± 0.05	0.16 ± 0.05	0.26 ± 0.08
DPA	22:5n-3		0.09 ± 0.02	0.08 ± 0.02	0.13 ± 0.04	0.09 ± 0.03	0.08 ± 0.02	0.05 ± 0.02	0.06 ± 0.02	0.11 ± 0.03
DHA	22:6n-3		0.92 ± 0.07	0.92 ± 0.07	1.08 ± 0.13	0.94 ± 0.13	0.79 ± 0.08	0.98 ± 0.11	0.87 ± 0.12	0.85 ± 0.10

Table 2. Relative proportion of plasma fatty acids groups (% of total fatty acid concentration), two biomarkers and the FA_{tot} (ng/µl) in urban, rural and natural habits, and from Plasmodium infected and uninfected juvenile house sparrows. Table shows mean values ± Standard Error. SFA: Saturated Fatty Acids; MUFA: Monounsaturated Fatty Acids; $PUFA_{tot}$: Total Polyunsaturated Fatty Acids; PI: Peroxidation Index; FA_{tot}: Total Fatty Acid Concentration (ng/µl).

	All		**Natural**	
Fatty acid class group / Biomarker	**Infected**	**Uninfected**	**Infected**	**Uninfected**
SFA	40.85 ± 0.46	41.21 ± 0.45	40.45 ± 0.84	40.24 ± 0.7
MUFA	28.42 ± 1.03	28.08 ± 0.99	25.20 ± 1.92	21.68 ± 1.83
ω-6 PUFA	28.63 ± 1.13	28.79 ± 1.13	31.60 ± 2.03	36.09 ± 1.95
ω-3 PUFA	1.90 ± 0.21	1.72 ± 0.17	2.57 ± 0.45	1.91 ± 0.42
$PUFA_{tot}$	30.73 ± 1.10	30.71 ± 1.11	34.35 ± 1.97	38.08 ± 1.89
ω-6/ω-3	29.17 ± 3.79	30.74 ± 3.41	22.23 ± 4.01	38.23 ± 5.94
PI	0.75 ± 0.02	0.76 ± 0.03	0.87 ± 0.04	0.93 ± 0.04
FA_{tot}	24.71 ± 0.77	24.81 ± 1.49	23.38 ± 1.09	22.33 ± 1.66

	Rural		**Urban**	
Fatty acid class group / Biomarker	**Infected**	**Uninfected**	**Infected**	**Uninfected**
SFA	41.71 ± 0.6	41.93 ± 0.7	40.4 ± 0.87	41.33 ± 0.87
MUFA	28.71 ± 1.35	30.37 ± 1.4	31.38 ± 1.83	31.20 ± 1.48
ω-6 PUFA	27.76 ± 1.55	25.87 ± 1.55	26.43 ± 2.11	25.54 ± 1.76
ω-3 PUFA	1.65 ± 0.32	1.59 ± 0.22	1.47 ± 0.24	1.69 ± 0.25
$PUFA_{tot}$	29.59 ± 1.45	27.70 ± 1.55	28.14 ± 2.02	27.47 ± 1.69
ω-6/ω-3	29.84 ± 4.75	21.60 ± 2.95	35.50 ± 9.25	33.33 ± 7.49
PI	0.71 ± 0.04	0.72 ± 0.04	0.69 ± 0.04	0.67 ± 0.04
FA_{tot}	22.96 ± 1.26	22.15 ± 1.11	27.59 ± 1.48	29.44 ± 3.69

The effect of habitat and infection on fatty acid composition

Results from the LMMs testing the effects of *Plasmodium* infection and habitat on the proportion of plasma FA groups are summarized in Table 3. *Plasmodium* infected birds had a significantly lower proportion of ω-6 PUFAs (χ^2=4.16, d.f.=1, *p*=0.04; Fig. 1) and a higher proportion of ω-3 PUFAs (χ^2=5.80, d.f.=1, *p*=0.02; Fig. 1) than uninfected individuals. Additionally, infected birds tended to have a higher proportion of MUFA and a lower overall proportion of PUFA_{tot} than uninfected ones, but these differences did not reach significance (MUFA: χ^2=3.01, d.f.=1, *p*=0.08; PUFA_{tot}: χ^2=2.97, d.f.=1, *p*=0.08; Fig. 1). Infection status was not associated to SFA (χ^2=0.14, d.f.=1, *p*=0.70). Moreover, a marginal effect was found regarding the interaction between infection status and habitat for the proportion of ω-3 PUFA (χ^2=5.68, d.f.=2, *p*=0.06), with infected birds showing higher proportion of ω-3 PUFA than uninfected birds in natural habitats (χ^2=5.8, d.f.=1, *p*=0.02; Fig. 2), but not in the urban (χ^2=0.12, d.f.=1, *p*=0.74; Fig. 2) or in the rural habitats (χ^2=0.32, d.f.=1, *p*=0.57; Fig. 2). The proportions of the other FA groups were not related to habitat nor the interaction between infection status and habitat (Table 3). Finally, the mean quantity of ω-3 PUFAs for uninfected and infected birds by *Plasmodium* was 0.43 (ng/µl) and 0.61 (ng/µl) in natural habitats, 0.49 (ng/µl) and 0.40 (ng/µl) in urban habitats, and 0.34 (ng/µl) and 0.39 (ng/µl) in rural habitats. The mean quantity of ω-6 PUFAs for uninfected and infected birds by *Plasmodium* was 7.99 (ng/µl) and 7.21 (ng/µl) in natural habitats, 7.75 (ng/µl) and 7.19 (ng/µl) in urban habitats, and 5.59 (ng/µl) and 6.27 (ng/µl) in rural habitats.

Table 3. Results of the Linear Mixed Models (LMM) analysing the effect of time (hour of capture), date (day of capture), body mass, *Plasmodium* infection status (Pla Inf: infected versus uninfected), habitat category (Habitat: urban, rural, and natural) and the interaction between infection status by *Plasmodium* and habitat (Pla Inf * Hab) on the proportion of the different groups of FA. Significant relationships ($p \leq 0.05$) are highlighted in bold. Conditional (and marginal) R^2 are shown. SFA: Saturated Fatty Acids, MUFA: Monounsaturated Fatty Acids, ω-6 PUFA: Omega 6 Polyunsaturated Fatty Acids, ω-3 PUFA: Omega 3 Polyunsaturated Fatty Acids, and PUFA$_{\text{tot}}$: Polyunsaturated Fatty Acids together.

	SFA			**MUFA**		
	χ^2	d.f.	*p*	χ^2	d.f.	*p*
Intercept	0.89	1	0.35	0.07	1	0.79
Time	6.35	1	**0.01**	2.27	1	0.13
Date	0.04	1	0.84	4.44	1	**0.03**
Body mass	2.01	1	0.16	0.69	1	0.41
Pla Inf	0.14	1	0.70	3.01	1	0.08
Habitat	0.99	2	0.61	4.24	2	0.12
Pla Inf *Hab	0.29	2	0.87	2.99	2	0.22
R^2	0.30 (0.08)			0.43 (0.17)		

	ω-6 PUFA			**ω-3 PUFA**			**PUFA$_{\text{tot}}$**		
	χ^2	d.f.	*p*	χ^2	d.f.	*p*	χ^2	d.f.	*p*
Intercept	14.17	1	**<0.001**	15.73	1	**<0.001**	13.17	1	**<0.001**
Time	5.63	1	**0.02**	0.52	1	0.47	6.52	1	**0.01**
Date	6.68	1	**0.01**	0.03	1	0.85	5.76	1	**0.02**
Body mass	1.98	1	0.16	0.10	1	0.75	2.38	1	0.12
Pla Inf	4.16	1	**0.04**	5.80	1	**0.02**	2.97	1	0.08
Habitat	2.58	2	0.28	0.30	2	0.86	3.26	2	0.20
Pla Inf *Hab	4.28	2	0.12	5.68	2	0.06	3.93	2	0.14
R^2	0.52 (0.18)			0.35 (0.05)			0.50 (0.2)		

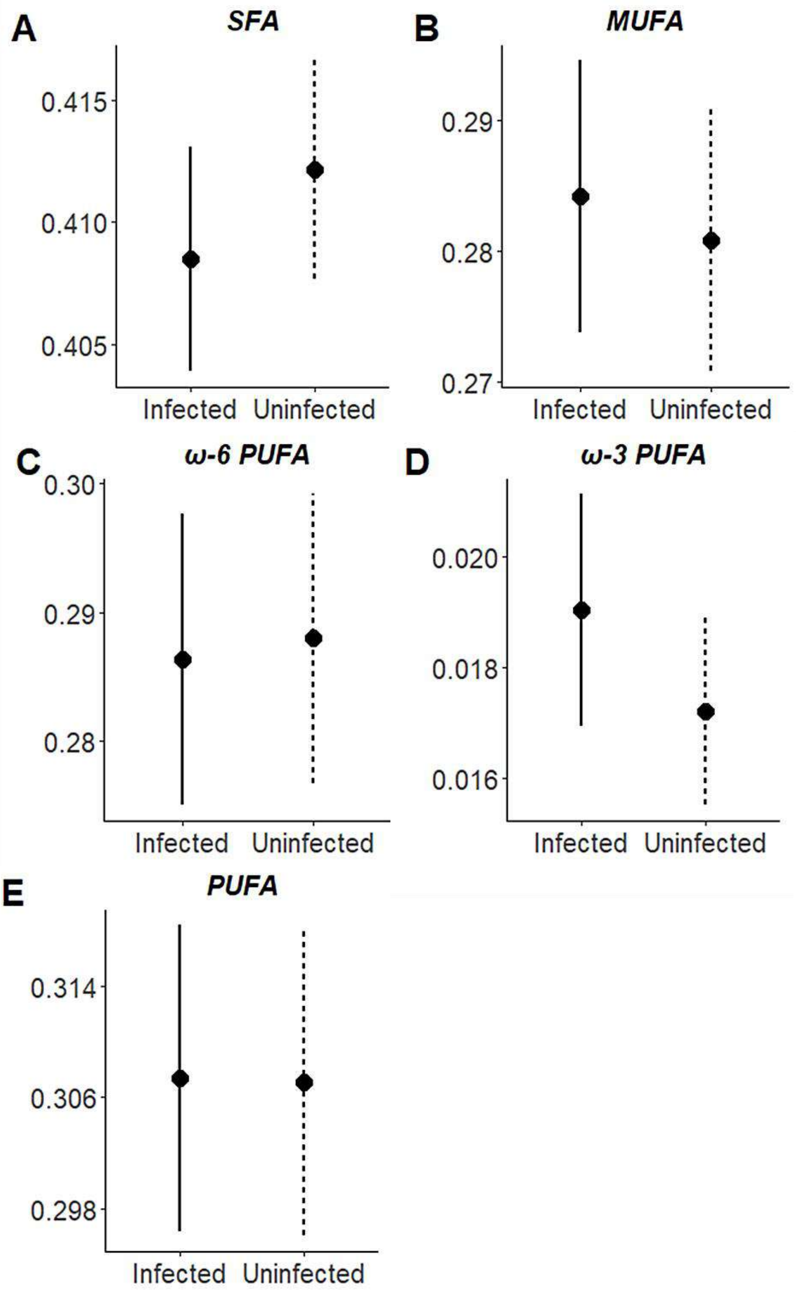


Figure 1. Mean relative proportion ± Standard Error of (A) saturated fatty acids (SFA), (B) monounsaturated fatty acids (MUFA), (C) omega-6 polyunsaturated fatty acids (ω-6 PUFA), (D) omega-3 polyunsaturated fatty acids (ω-3 PUFA) and (D) polyunsaturated fatty acids ($PUFA_{tot}$) for juvenile house sparrows either infected or uninfected by *Plasmodium*.

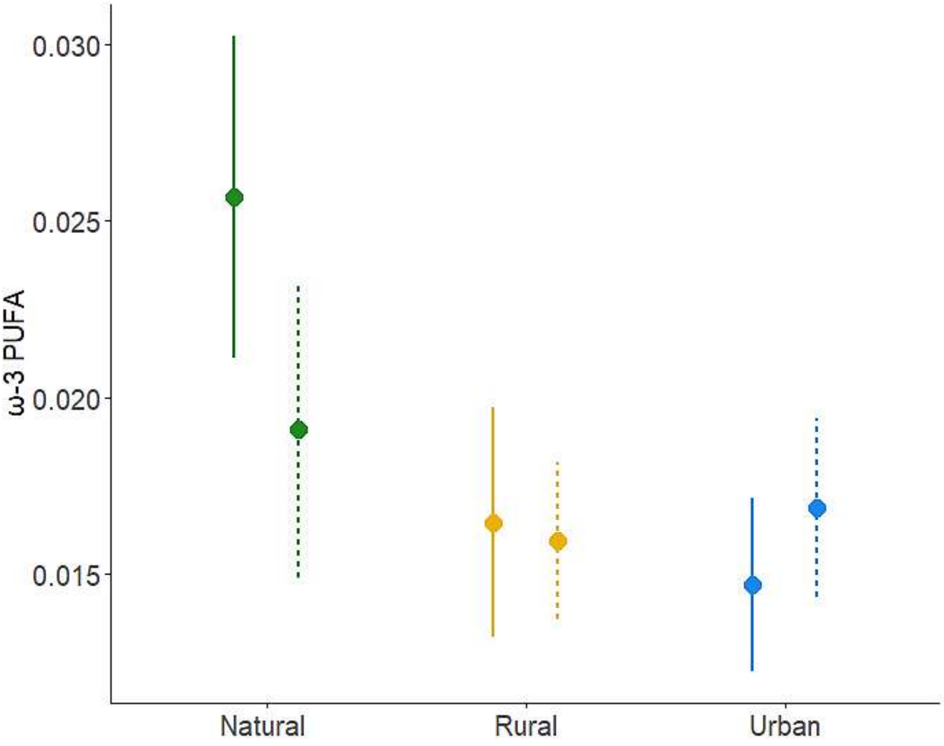


Figure 2. Mean proportion ± Standard Error of omega-3 polyunsaturated fatty acids (ω-3 PUFA) of *Plasmodium* infected (straight line) and uninfected birds (dotted line) in natural, rural, and urban habitats.

The effect of habitat and infection on estimated biomarkers and FA$_{tot}$ levels

Results for LMMs analyses testing the effects of *Plasmodium* infection and habitat on the biomarkers and FA$_{tot}$ are summarized in Table 4. The ω-6/ω-3 ratio differed significantly with the infection status of birds (χ^2=8.7, d.f.=1, *p*=0.003) and the interaction between the infection status and habitat category (χ^2=8.28, d.f.=2, *p*=0.02). *Plasmodium* infected birds had lower ω-6/ω-3 ratio levels than uninfected individuals in natural habitats (χ^2=8.69, d.f.=1, *p*=0.003; Fig. 3), but there are no significant differences in birds from rural (χ^2=0.70, d.f.=1, *p*=0.40; Fig. 3) or urban habitats (χ^2=0.01, d.f.=1, *p*=0.93; Fig. 3). The FA$_{tot}$ was only significantly associated with the habitat category (χ^2=7.12, d.f.=2, *p*=0.03), with urban individuals showing a higher amount of FA concentration than rural birds (Est=0.09, S.E.=0.04, *z* value=2.59; *p*=0.03; Fig. 4), but there were no significant differences between urban and natural habitats (Est=0.07, S.E.=0.04, *z* value=1.79; *p*=0.17) nor between rural and natural habitats (Est=-0.02, S.E.=0.04, *z* value=-0.64; *p*=0.79). In addition, we found marginally significant differences in PI between habitat categories (χ^2=5.32, d.f.=2, *p*=0.07), where birds from rural habitats had lower PI values than conspecifics from the natural habitat (Est=-0.16, S.E.=0.07, *z* value=-2.15, *p*=0.08; Fig. S2), but this differences were not significant between urban-natural (Est=-0.14, S.E.=0.07, *z* value=-1.85, *p*=0.15; Fig. S2) nor between urban-rural habitats (Est=0.02, S.E.=0.07, *z* value=0.28, *p*=0.96; Fig. S2). Finally, marginally significant differences in PI between the infection status, where the PI tended to be lower in *Plasmodium* infected than in uninfected birds (χ^2= 2.98, d.f.=1, *p*=0.08; Fig. S2).

Table 4. Results of the Linear Mixed Models (LMM) analysing the effect of time (hour of capture), date (day of capture), body mass, infection status by *Plasmodium* (Pla Inf: infected versus uninfected), habitat category (Habitat: urban, rural and natural) and the interaction between infection status by *Plasmodium* and habitat (Pla Inf * Hab) on different biomarkers of fatty acids. Significant relationships ($p \leq 0.05$) are highlighted in bold. Conditional (and marginal) R^2 are shown. PI: Peroxidation Index, FA$_{tot}$: Total Fatty Acid concentration.

	PI			**ω-6/ω-3 ratio**			**FA$_{tot}$**		
	χ^2	d.f.	*p*	χ^2	d.f.	*p*	χ^2	d.f.	*p*
Intercept	9.24	1	**<0.001**	3.08	1	0.08	724.77	1	**<0.001**
Time	32.59	1	**<0.001**	0.02	1	0.89	12.53	1	**<0.001**
Date	1.51	1	0.22	2.97	1	0.08	0.15	1	0.69
Body mass	1.35	1	0.25	0.01	1	0.919	1.05	1	0.30
Pla Inf	2.98	1	0.08	8.70	1	**0.003**	0.88	1	0.35
Habitat	5.32	2	0.07	0.67	2	0.72	7.12	2	**0.03**
Pla Inf * Hab	2.16	2	0.34	8.28	2	**0.02**	0.63	2	0.73
R^2	0.48 (0.35)			0.49 (0.05)			0.19 (0.17)		

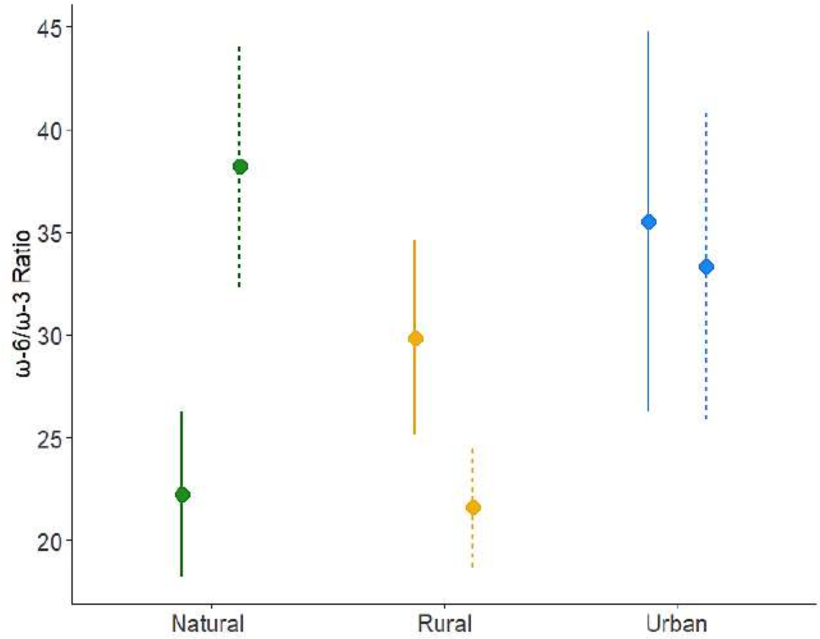


Figure 3. Mean ratio of ω-6 and ω-3 polyunsaturated fatty acids (PUFAs) ± Standard Error of Plasmodium infected (straight line) and uninfected birds (dotted line) in natural (green), rural (yellow), and urban (blue) habitats.

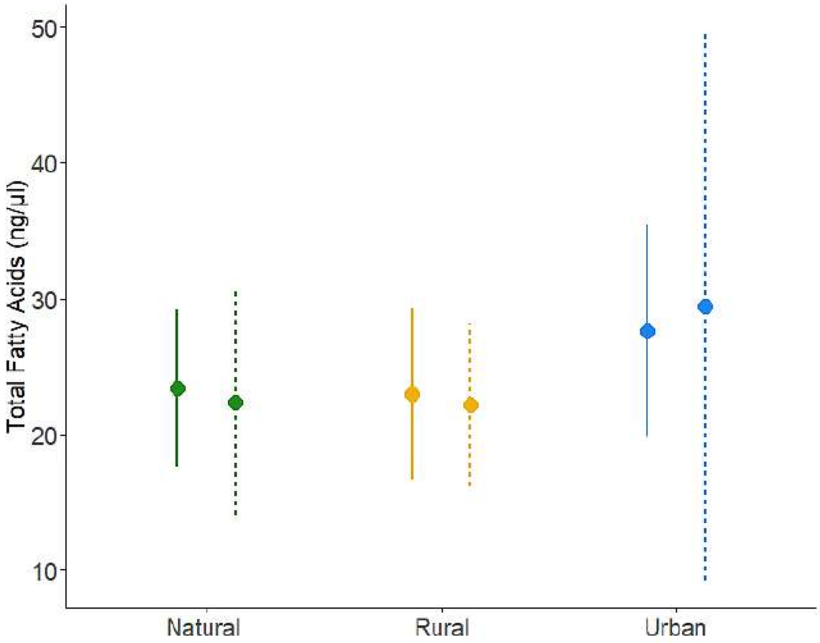


Figure 4. Mean total fatty acid concentration (ng/µl) ± Standard Error, in Plasmodium infected (straight line) or uninfected (dotted line) house sparrows captured in natural (green), rural (yellow), and urban (blue) habitats.

Discussion

Here, we investigate how serum fatty acid composition in juvenile house sparrows is related to urbanization level and malaria infection. The main results revealed that *Plasmodium* infected birds had higher proportion of ω-3 PUFA, but lower proportion of ω-6 PUFA than uninfected birds. This was also reflected in a lower ω-6/ω-3 ratio in the infected birds, but only in natural habitats. The FA_{tot} was higher in urban birds compared to birds from rural habitats, independently of their infection status. Finally, the PI tended to be higher in natural habitats compared to rural or urban habitats, and lower in infected birds by *Plasmodium* than uninfected ones.

Fatty acid composition in relation to infection status

Several studies have documented negative effects on fitness or fitness-related traits when infected by avian malaria (Asghar et al., 2015; Atkinson et al., 1991; Jiménez-Peñuela et al., 2019; Lachish et al., 2011; Martínez-de la Puente et al., 2010; Marzal et al., 2008, 2005). Yet, the links between FA composition and avian malaria infection is so far unexplored, despite the known effects of PUFAs on immune responses, its environmental dependence and fitness effects, especially in juveniles (Twining et al., 2020, 2018). Here, we show that ω-6 PUFA was relatively lower and ω-3 PUFAs relatively higher in infected juvenile birds compared to uninfected ones. This is interesting given the two different immune pathways that these PUFAs are involved in. During infection or other inflammatory processes, ω-6 PUFA are metabolized to pro-inflammatory prostaglandins, while the down-stream immune components of the ω-3 PUFA pathway is anti-inflammatory (Alagawany et al., 2019; Chang et al., 2018; Hulbert et al., 2005; Simopoulos, 2011). Both circulating ω-6 and ω-3 PUFAs are influenced by dietary intake as well as by physiological processes, such as metabolism, elongation, selective transportation and use (Alagawany et al., 2019; Hulbert et al., 2005; Hulbert and Abbott, 2012; Price and Valencak, 2013).

However, PUFA elongation is rather inefficient and ingested PUFAs usually maintain their original structure, which make them useful as dietary tracers (Twining et al., 2016). Thus, the higher relative proportion of ω-3 PUFAs found in infected birds may suggest that they have preferentially consumed and/or mobilized them from storage to promote an anti-inflammatory immune response towards *Plasmodium* infection. Indeed, the consumption of rich food in ω-3 PUFAs have previously been shown to be beneficial during active inflammation to improve the immune response without increasing the oxidative stress (Alagawany et al., 2019). Moreover, and in line with this, we also found significant lower proportions of ω-6 PUFAs and a marginally lower PI in *Plasmodium* infected birds compared to uninfected ones. These results could be interpreted as the infected birds cannot afford the joint negative effects of *Plasmodium* infection and the increment of oxidative stress derived from the pro-inflammatory immune response generated by ω-6 PUFAs and the corresponding lipid peroxidation. Alternatively, the higher proportion of ω-3 PUFAs and lower proportion of ω-6 PUFAs could indicate that birds are more susceptible to infection possibly because less pro-oxidants are produced to attack the parasites. A controlled experiment will be useful to disentangle the causal effects of these results. Independently of the cause, this fact makes the ω-6/ω-3 ratio especially sensible to the variation of these two PUFAs, where a low ω-6/ω-3 ratio is either a result of a low proportion of ω-6, or of a high proportion of ω-3. Nevertheless, since the relative abundance in relation to each other is what determines which immune pathway is activated, the ω-6/ω-3 ratio is potentially affecting more the long-term costs of defeating an infection rather than the proportion of the individual PUFA groups (Pamplona et al., 2002).

Fatty acid composition in relation to urbanization

The FA_{tot} was significantly higher in birds from the urban habitats compared to those from rural habitats. This suggest that the availability of fat-rich food for house sparrows are higher in urban habitats compared to rural habitats (Heiss et al., 2009; Mckinney, 2002; Meyrier et al., 2017). However, there was no significant differences between urban and natural environments, nor between rural and natural habitat. Moreover, despite the mentioned benefits of PUFAs, they are highly susceptible to lipid peroxidation under conditions of high oxidative stress (Isaksson et al., 2017; Pamplona et al., 2002). Thus, the more long-chained PUFAs, the higher PI we could expect. Although, it did not reach significance, there was a tendency for higher PI in birds from natural habitats compared with their urban and rural conspecifics. In contrast, another study showed that two sparrow species from urban habitats had a higher PI than their rural conspecifics, linked to a major susceptibility to lipid peroxidation and higher oxidative stress in birds of those altered environments (Isaksson et al., 2017). Unfortunately, we were not able to measure lipid peroxidation here, so we cannot directly test if urban birds suffer from increased oxidative damage and therefore have slightly less PUFAs available. Thus, our results could be reflecting that those birds under situations with increased oxidative stress, are not able to afford the joint negative effects of high PI levels and anthropic conditions urbanization or *Plasmodium* infections disease.

Fatty acid composition in relation to the interaction of infection status and urbanization

Our results revealed that the ω-6/ω-3 ratio was lower in the *Plasmodium* infected birds compared to the uninfected birds when living in natural habitats but not when living in urban or rural habitats. This result is likely to be driven by the slight habitat difference in the relative proportion of ω-3 PUFAs among the infected versus the uninfected birds, with infected birds from the natural habitats having higher ω-3 PUFAs. Indeed, these differences can be noted also in the quantity of ω-3 PUFAs, with infected birds from natural habitats having higher absolute levels. Thus, our results may indicate that infected birds living in higher anthropized habitats could have limited availability of ω-3-rich food to consume compared to birds from natural habitats. This has also been shown for other bird species (Andersson et al., 2015; Isaksson et al., 2017). Having access to highly nutritional food has been shown to mount a higher immune response and higher membrane resistance during *Plasmodium* infection compared to control birds (Delhaye et al., 2018). Thus, juvenile house sparrows from natural habitats may be better in combating the *Plasmodium* infection off compared to the infected birds in the urban and rural habitat.

Alternatively, it is also possible that birds from more anthropized environments already metabolized the ω-3 PUFAs to immune components for other reasons (e.g., environmental stressors such as exposure to chemical and/or light pollution) leaving less circulating ω-3 PUFAs to other important functions. Thus, growing up in anthropized habitats and being infected could have greater costs due to the exposure to multiple stressors and limited resources. Our results highlight a differential availability/mobilization of ω-3 in infected compared to uninfected birds from natural habitats while rural and urban birds did not show this pattern. The negative and potentially synergic effects of anthropization and *Plasmodium* infection was previously highlighted in a study of house sparrows from the same habitats. There it was suggested that birds in poor boy condition were less likely to survive when infected by *Plasmodium* in urban habitats (Jiménez-Peñuela et al., 2019). Our present results further support this by showing a FA profile for urban juvenile birds that potentially make it more challenging to handle *Plasmodium* infection.

Conclusion

The present results highlight the importance of studying bird's physiological condition and nutritional status when considering the impacts of disease in wildlife populations. The results imply that infected birds, especially from natural habitats, have a composition of PUFAs more beneficial for mounting an anti-inflammatory immune response, whereas the infected birds from more anthropized habitats have a pro-inflammatory PUFA composition. This may provide a mechanistic link and explanation for the negative relationship between house sparrows decline in the cities and *Plasmodium* infections dynamics. Diet supplementation or the improvement of anthropized habitats with a more heterogenic landscape where birds can eat seeds, cereals, and insects, may provide a non-invasive approach to reduce the impact of *Plasmodium* infection and improve the general health of urban birds. This may be especially important at early life stages and would be interesting to further investigate in the future. Our results shed some light into how anthropic activities could determine the capacity of wildlife to fought off infectious diseases, affecting their health and their populations demography.

Ethics Statement

Bird trapping was carried out with all the necessary permits from Consejería de Medio Ambiente, and Consejería de Agricultura, Pesca y Desarrollo Rural (Junta de Andalucía) and bird sampling on private land and in private residential areas were conducted with all the necessary permits and consent of owners. The CSIC Ethics Committee approved the experimental procedures on 9 March 2012.

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Availability of data and materials

The data supporting the conclusions of this article are included within the article. The datasets used and/or analyzed during the present study will be made available by the corresponding author upon reasonable request.

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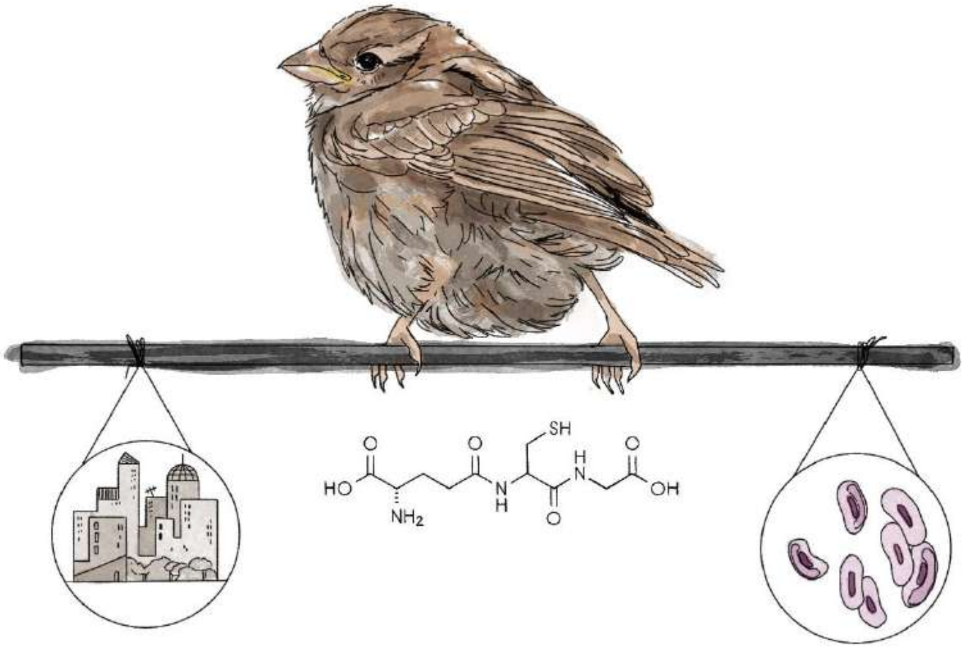
CHAPTER 4.

OXIDATIVE STRESS IN HOUSE SPARROWS IN RELATION TO HAEMOSPORIDIAN INFECTION ALONG AN URBANIZATION GRADIENT



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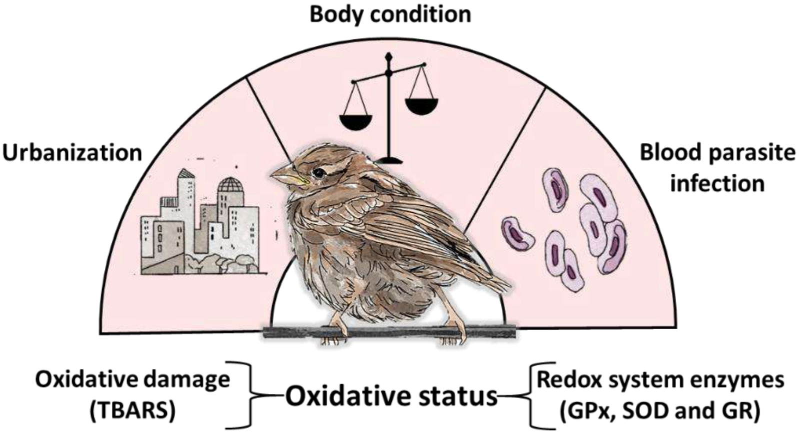
Graphical abstract



Highlights

- Four biomarkers of the oxidative status of wild house sparrows were analysed with respect to the habitat they live in and their parasite infection status.
- Birds from urban habitats had higher levels of oxidative damage to lipids measured as TBARS levels.
- Infections by the avian malaria parasite *Plasmodium* affected the redox system of wild birds, influencing the activity of three antioxidant enzymes (GPx, SOD and GR).
- Birds with lower body condition showed higher evidence of oxidative damage in urban habitats, while the opposite occurred in birds from rural and natural habitats.
- The body condition of birds may modulate the levels of oxidative stress as is related with oxidative damage levels differently in urban and non-urban habitats and with the antioxidant enzymatic activity against stressors.

Keywords: Antioxidant enzymes, avian malaria, *Haemoproteus*, *Leucocytozoon*, oxidative damage, *Passer domesticus*, *Plasmodium*.

Abstract

Living organisms are exposed to a diversity of stress sources. The oxidative stress occurs when the equilibrium between Reactive oxygen substances (ROS), that can cause oxidative damage to biomolecules, and the activity of antioxidant substances is lost. Both urbanization and parasite infections are important sources of ROS that cause different and severe deleterious effects on wildlife. Nevertheless, their joint effects, especially with the increment of landscape anthropization, is still unclear. Here, we analysed the oxidative stress of wild juvenile male house sparrows (*Passer domesticus*) with respect to their infection status by three common blood parasite genera (namely *Plasmodium*, *Haemoproteus* or *Leucocytozoon*), along an urbanization gradient. We analysed samples from 689 birds captured during 2013 in 45 localities grouped in 15 triplets including one urban, one rural, and one natural habitat per triplet. We measured the levels of i) thiobarbituric acid reactive substances (TBARS) as an indicator of the oxidative damage to lipids and the activity of the ii) glutathione peroxidase (GPx), iii) superoxide dismutase (SOD), and iv) glutathione reductase (GR) enzymes as indicators of bird´s antioxidant capacity. The level of TBARS were higher in birds from urban than rural habitats. Also, the relationship between TBARS with bird body condition was positive in birds from rural and natural habitats but negative in birds from urban habitats. The activity of GPx, SOD and GR was lower in *Plasmodium* infected birds than in uninfected ones. Finally, individuals in better body condition showed higher activity of GPx and SOD but lower activity of GR. Overall, these results suggest that both urbanization and parasite infection affect the oxidative status of wild house sparrows and highlight the importance of birds body condition in their physiological responses against stressors.

Introduction

Reactive oxygen species (ROS) is a collective term to include different chemical substances with distinct origins that may cause oxidative damage to biomolecules such as proteins, lipids, or DNA (Li et al., 2016). ROS are produced, among others, as by-products of basal metabolism and during inflammatory responses (Costantini, 2019; Isaksson, 2015; Li et al., 2016; Sorci and Faivre, 2009). Organisms have evolved antioxidant systems, with both endogenous enzymes and external non-enzymatic compounds (i.e., vitamins from dietary sources), that prevent, reduce, and repair ROS-induced damage (Costantini and Verhulst, 2009; Li et al., 2016; Sorci and Faivre, 2009). When the balance with antioxidants and pro-oxidant is lost, either with an increase of ROS or with a decrease of antioxidant activity, the oxidative stress increases producing damage to biomolecules (Costantini and Verhulst, 2009; Li et al., 2016).

During the course of infection, organisms may develop inflammatory responses where cells of the immune system produce cytotoxic compounds that act like ROS (Sorci et al., 2013; Sorci and Faivre, 2009). These are non-specific physiological responses that can also harm the host and develop into chronic inflammation (Costantini and Verhulst, 2009; Sorci and Faivre, 2009). Even inflammatory responses can be costly, individuals unavailable to produce these responses are usually more susceptible to suffer increased infection-induced mortality (Sorci and Faivre, 2009). Correlative and experimental studies have confirmed that parasites are an important source of physiological stress (del Cerro et al., 2010; Martínez-de la Puente et al., 2011; Merino et al., 1998; Videvall et al., 2015). Overall, avian malaria parasites of the genus *Plasmodium*, and the related malaria-like genera *Haemoproteus* and *Leucocytozoon*, are widespread blood parasites with negative impacts on the physiological condition of birds (Atkinson et al., 1991; Valkiūnas, 2005). Different deleterious effects of blood parasite infections on diverse life-history traits of birds have been reported. Infected individuals from a variety of avian species suffer, among others, negative impacts on health (Marzal et al., 2008; Merino et al., 2000), survival (Martínez-de la Puente et al., 2010), reproductive success (Cuevas et al., 2021; Marzal et al., 2005; Merino et al., 2000), hormone modulation (Names et al., 2021) and altered responses of oxidative stress (Arriero and Møller, 2008; Martínez-de la Puente et al., 2011; Morales et al., 2006). However, other studies have not found any detrimental consequences of haemosporidian infections on body condition, immunocompetence, plasma corticosterone concentrations, total antioxidant capacity, or reactive oxygen metabolites in red-winged blackbirds (Schoenle et al., 2017). Environmental and individuals extrinsic and intrinsic factors will determine the susceptibility of the host to be infected, the capacity to modulate immune responses to fight infections, and the potential costs of these responses (Delhaye et al., 2018), likely causing such discrepancies.

The continuous increment of urbanization has created new habitats where only a reduced number of species able to exploit anthropic resources, are competent to survive (Mckinney, 2002; Sol et al., 2014). The detrimental effects of habitat anthropization on birds life history have been reported in different urban dwelling species. Urbanization negatively affects body size and condition of wild birds (Jiménez-Peñuela et al., 2019; Liker et al., 2008), as well as their reproductive success and survival (Chamberlain et al., 2009; Sepp et al., 2018; Sumasgutner et al., 2014). In addition, telomere attrition rate was higher in birds breeding in urban than in natural habitats (Ibáñez-Álamo et al., 2018; Salmón et al., 2016; Watson et al., 2017). These impacts may be caused by the availability and quality of the food consumed (Costantini, 2010; Delhaye et al., 2018; Romieu et al., 2008) and the presence of pollutants or other environmental stressors in cities (Bichet et al., 2013; Herrera-Dueñas et al., 2014; Koivula et al., 2011; Romieu et al., 2008; Salmón et al., 2018). Pollutants such as NO_x, SO_2 or O_3, derived from the burning of fossil fuels, are toxic and deleterious substances that have been demonstrated to increase the oxidative stress of birds living in urban habitats (Herrera-Dueñas et al., 2017; Isaksson, 2010; Koivula et al., 2011; Salmón et al., 2018). Nevertheless, contradictory results have also frequently been reported. For example, no significant differences in the levels of physiological stress was found in birds from urban compared to natural populations nor across habitats with different urbanization levels (Chávez-Zichinelli et al., 2010; Ibáñez-Álamo et al., 2020). Thus, the effect of parasite infection, habitat anthropization and therefore, their joint effects on bird oxidative stress, is still far from clear.

In this study, we test the impact of two potential sources of physiological stress such as urbanization and parasite infection, as well as their interaction, on the oxidative status of wild birds. We evaluated both the oxidative damage and the antioxidant capacity of birds uninfected and infected by *Plasmodium*, *Haemoproteus* or *Leucocytozoon* parasites, from populations along an urbanization gradient considering urban, rural, and natural habitats. The house sparrow (*Passer domesticus*) is a widespread, abundant, and sedentary urban exploiter species that has lived in contact with humans for centuries, taking advantage of anthropogenic food resources and cavities for nesting (Anderson, 2007; De Laet and Summers-Smith, 2007; Hanson et al., 2020; Mckinney, 2002). This passerine is currently undergoing a dramatic population decline, especially in urban areas where nutritional constrains, intensive and large-scale farming and agriculture, pollution, pesticides and infectious diseases such as avian malaria, among others, have been suggested as the main causes of its decline (Bichet et al., 2013; Dadam et al., 2019; Herrera-Dueñas et al., 2017, 2014; Meillère et al., 2017; Meyrier et al., 2017).

Material and methods

Study area and sampling design

Fieldwork was conducted at 45 localities in the province of Huelva, Seville, and Cadiz, in southern Spain. Sampling localities were grouped into 15 geographically close triplets, formed by one urban (defined as a more densely populated area), one rural (characterized by high density of livestock), and one natural habitat (areas better preserved from human activities and where wildlife predominated over both livestock and humans). The categorization of the localities was based on visual inspection of the habitat (for further information on the sampling design see Ferraguti et al., 2016). The average distance within localities of the same triplet was 5875.19 m (±S.D. 4584.28 m). Overall, house sparrows have a home range radius of 1-2 km with < 10% of individuals dispersing over that distance (Snow and Perrins, 1998; Summers-Smith, 1963, 1956), consequently we assume a reduced mobility of individuals between localities that still allow gene flow.

Bird sampling

From July to October of 2013, overlapping with higher density of juvenile house sparrows in the area, birds were captured using mist-nets and bird call playbacks. Birds were ringed with a numbered metal ring, sexed and aged when possible, according to their plumage characteristics and skull ossification (Svensson, 2009). Birds were classified as juveniles (i.e., those born in the calendar year, Euring code 3), adults (i.e., those born before the actual calendar year, Euring code 4) or unknown age (i.e., birds for which it was not possible to differentiate between these two categories, Euring code 2). In addition, bird body mass and wing length were measured with an electric balance (digital scale to the nearest 0.1 g) and a metal ruler (to the nearest 0.5 mm), respectively. Both variables were used to estimate individual body condition according to Peig and Green (2009). Subsequently, birds were blood sampled from the jugular vein with a sterile syringe, never exceeding 1% of the body mass. The birds were released at the same place of capture without any apparent damage. Blood samples were transferred to Eppendorf tubes and stored in cold-boxes during the fieldwork and kept at 4ºC until centrifugation the next morning for 10 min at 1700g (4000 rpm) to separate serum and cellular fractions. The latter was frozen at -20ºC until further analyses. In this study, we only included samples from juvenile birds (Euring code 3) as they are exposed to the environmental conditions during the reduced period of their life, and may be recently infected during the nestling or post-fledgling period (Cosgrove et al., 2008). Due the potential sex differences in the responses to oxidative damage and antioxidant production (Costantini, 2010), only male birds were analysed. Finally, one of the triplets was excluded from the analyses due to the lack of blood samples of sufficient volume from male juveniles in one of the localities.

Molecular analyses

Genomic DNA was extracted from the blood cellular fraction using the Maxwell®16 LEV System Research (Promega, Madison, WI). The sex of those individuals that could not be clarified in the field according to plumage characteristics was molecularly determined following Griffiths et al., (1998, 1996). The infection status and the identity of parasite genus was assessed through the amplification of a fragment of the cytochrome *b* gene following Hellgren et al., (2004). Negative samples were repeated twice to avoid the occurrence of false negatives (McClintock et al., 2010). Both negative controls for PCR reactions (at least one per plate) and DNA extractions (one per 15 samples) were included in the analyses. PCR amplifications were resolved in 1.2% agarose gels and positive amplifications were sequenced unidirectionally (Macrogen Inc.; Amsterdam, The Netherlands). Sequences were edited using Sequencher™ v.4.9 (Gene Codes Corp. © 1991–2009, Ann Arbor, MI 48108, USA) and parasite genus identified by comparison with sequences deposited in GenBank (National Center for Biotechnology Information) and MalAvi databases (Bensch et al., 2009). Information of birds parasite infections was previously analysed in the context of other studies (Ferraguti et al., 2018; Jiménez-Peñuela et al., 2019).

Biochemical analyses

Measuring oxidative stress in blood provides an integrated measure of the organism oxidative status and its variability, especially when the experienced stress or exposure is low to moderate, and chronic rather than acute (Costantini and Verhulst, 2009; Isaksson and Bonier, 2020; Ouyang et al., 2018). The concentrations of one marker of oxidative damage and three antioxidant enzymes were determined from the blood cellular fraction of the house sparrows at the Ecophysiology Laboratory at the Doñana Biological Station (LEF-EBD-CSIC).

Blood cells were mixed with miliQ water (1:4 proportion) and centrifuged at 14,000 rpm during 30 min at 4ºC to lyse the cells. The supernatant was separated and kept at -80ºC until each respective biomarker analyses.

The oxidative damage caused by oxidant agents to lipids, especially to unsaturated ones, produce metabolites from lipid peroxidation called malondialdehyde (MDA) (Buege and Aust, 1978; Costantini, 2019). We use thiobarbituric acid reactive substances or TBARS (nmol MDA/ml) as the biomarker to quantify this reaction. When the MDA reacts with the thiobarbituric acid produces red-pink products that can be measured at a wavelength of 535 nm (Buege and Aust, 1978). The antioxidant capacity of birds was measured by quantifying the activity of three enzymes that takes part in the glutathione (GSH) redox system (Isaksson, 2010). The superoxide dismutase (SOD) is involved in a crucial cellular antioxidant defence catalysing the dismutation of the superoxide anion (O_2^-) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) (Arthur and Boyne, 1985). SOD (U/mg of protein) activity was determined with the inhibition rate of xanthine oxidase (XOD) as the source of peroxide radicals. The catalysis of XOD produces red products that were measured at a wavelength of 550 nm (Arthur and Boyne, 1985; Suttle, 1986; Suttle and McMurray, 1983; Wooliams et al., 1983). The glutathione peroxidase (GPx) catalyses the reduction of both organic peroxides (R-OOH, caused by the oxidation of organic macromolecules with ROS), and hydrogen peroxide (H_2O_2, caused by the activity of SOD), into their corresponding stable alcohols (R-OH) and water. The GPx catalases the transformation of glutathione (GSH) into oxidised glutathione (GSSG) (Paglia and Valentine, 1967). Finally, the glutathione reductase (GR) reduces the GSSG into GSH by oxidising NADPH to $NADP^+$ to immediately counteract the activity of the GPx (Melissinos et al., 1981). The activities of GPx and GR were quantified by the decrease in the absorbance at a wavelength of 340 nm due to the oxidation of NADPH to $NADP^+$ (Goldberg and Spooner, 1983; Melissinos et al., 1981; Paglia and Valentine, 1967). All methods implemented to detect SOD, GPx and GR were adapted to the autoanalyzer "Cobas Integra 400" and the concentration of each biomarker was referred to the total protein content expressed in mg/ml.

Statistical analyses

A Principal components analysis (PCA) was performed using a correlation matrix to summarize the antioxidant activity of SOD, GPx and GR enzymes into two principal components (PC1 and PC2). Variables were logarithmically transformed, centred, and scaled prior to the PCA. We independently analysed TBARS with respect to SOD, GPx and GR, because they reflect different aspects of the oxidative status of birds. TBARS is a biomarker that measures the oxidant reactions of ROS with lipids and their consequent oxidative damage, while SOD, GPx, and GR refer to the enzymatic antioxidant capacity from the GSH redox system of birds. The effects of landscape use and parasite infections on bird oxidative status were tested by fitting Linear Mixed-Effects Models (LMM). TBARS levels, PC1 and PC2 (continuous) were included as dependent variables in separate models. Time and date of sampling (continuous), body condition (continuous), habitat category (categorial: urban, rural, and natural), *Plasmodium*, *Haemoproteus* and *Leucocytozoon* infection status (categorical: infected/uninfected by each parasite), and the interaction between body condition and habitat were included as independent variables. Sampling locality nested into triplet were included as a random factor to control for the geographical stratification of the study design. A backwards stepwise selection procedure was applied by removing either the variable or the interaction with the highest *p* values. This procedure was used until obtaining a simplified model including variables with $p \leq 0.10$. When the interaction was retained in the model, both variables included in the interaction were also maintained. Differences between the three factor levels of habitat category were tested with Tukey's post-hoc test when necessary. For each model, the marginal (considering only fixed factors) and conditional (considering fixed and random factors) variance explained (R^2) were calculated (Nakagawa and Schielzeth, 2013). Collinearity between independent variables was tested with the Variance Inflation Factor (VIF) (Zuur et al., 2010) and no evidence of collinearity (values < 4) was found. Normality of continuous variables, as well as the residuals of all the LMMs, were tested by checking normality qq-plots. Initially, 689 individuals were included in the analyses, but due to the samples of blood cellular fraction from some individuals did not reach enough quantity to test all the enzymes, sample size differed between analyses (TBARS models, N = 600; GPx, GR and SOD models, N = 488). Models were run with the higher sample size available for each analysis. All statistical analyses were conducted in R (R version 4.0.4 "Lost Library Book"; R Foundation for Statistical Computing 2021) using the packages: *arm*, *car*, *cowplot*, *emmeans*, *ggplot2*, *Hmisc*, *lattice*, *lme4*, *lmerTest*, *lsmeans*, *MASS*, *Matrix*, *multcomp*, *MuMIn*, *nlme*, *phia*, *RcppEigen* and *stats*.

Results

Body condition (Estimate ± S.E. = 0.001 ± 0.004, $\chi^2 = 0.07$, d.f. = 1, $p = 0.79$), habitat ($\chi^2 = 5.58$, d.f. = 2, $p = 0.06$) and their interaction ($\chi^2 = 5.59$, d.f. = 2, $p = 0.06$) were the only variables retained in the TBARS models ($R^2m = 0.01$, $R^2c = 0.29$). Birds from urban areas tended to have higher TBARS levels than birds from rural habitats (Estimate ± S.E. = 0.30 ± 0.13, z = 2.34, $p = 0.05$), while no significant differences were found between rural-natural habitats (0.21 ± 0.13, z = -1.56, $p = 0.26$) and neither between birds from urban-natural habitats (0.09 ± 0.12, z = 0.71, $p = 0.76$). Moreover, TBARS was marginally and positively related with body condition in natural and rural habitats but negatively related in urban habitats (Fig.1).

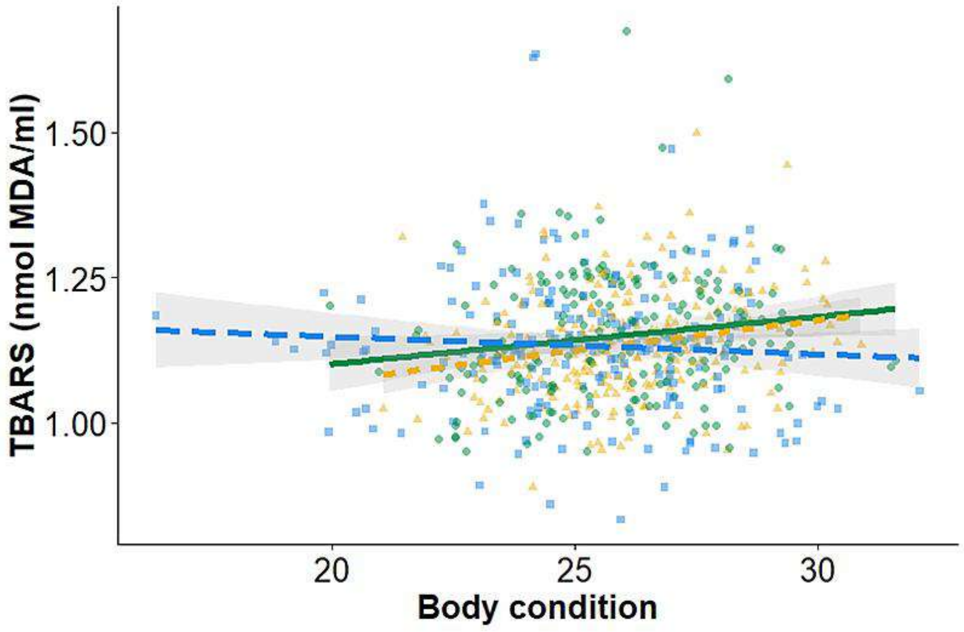


Figure 1. Relationship between TBARS levels and the body condition of birds from natural (green straight line and circles), rural (yellow dotted line and triangles) and urban (punctuated and blue line and squares) habitats.

The two principal components of the PCA calculated over the correlation matrix of SOD, GPx and GR enzymes, explained 82.68% of the variance. The PC1 had an eigenvalue of 1.6 and explained 53.21% of the variance, while the PC2 had an eigenvalue of 0.9 and explained 29.5% of the variance. The contribution of GPx, GR and SOD to PC1 was 39.05, 14.47 and 43.48, and to the PC2 was 16.44, 80.2, 3.65, respectively. The loadings for GPx, GR and SOD were 0.62, 0.42 and 0.66 of the PC1 and -0.41, 0.89 and -0.18 of the PC2 (Fig.2). PC1 ($R^2c = 0.43$, $R^2m = 0.04$) was significantly lower in birds infected by *Plasmodium* (-0.22 ± 0.10, $\chi^2 = 4.69$, d.f. = 1, $p = 0.03$) and tended to be higher in individuals infected by *Leucocytozoon*, although the last association was only marginally significant (0.20 ± 0.11, $\chi^2 = 3.21$, d.f. = 1, $p = 0.07$) (Fig.3). PC2 ($R^2c = 0.26$, $R^2m = 0.07$) was positively associated with the hour of capture (1.05 ± 0.32, $\chi^2 = 10.48$, d.f. = 1, $p = 0.001$) and negatively with the date of capture (-0.006 ± 0.002, $\chi^2 = 11.24$, d.f. =

1, *p* = 0.001) and with the body condition of birds (-0.04 ± 0.02, χ^2 = 3.94, d.f. = 1, *p* = 0.047; Fig. 4).

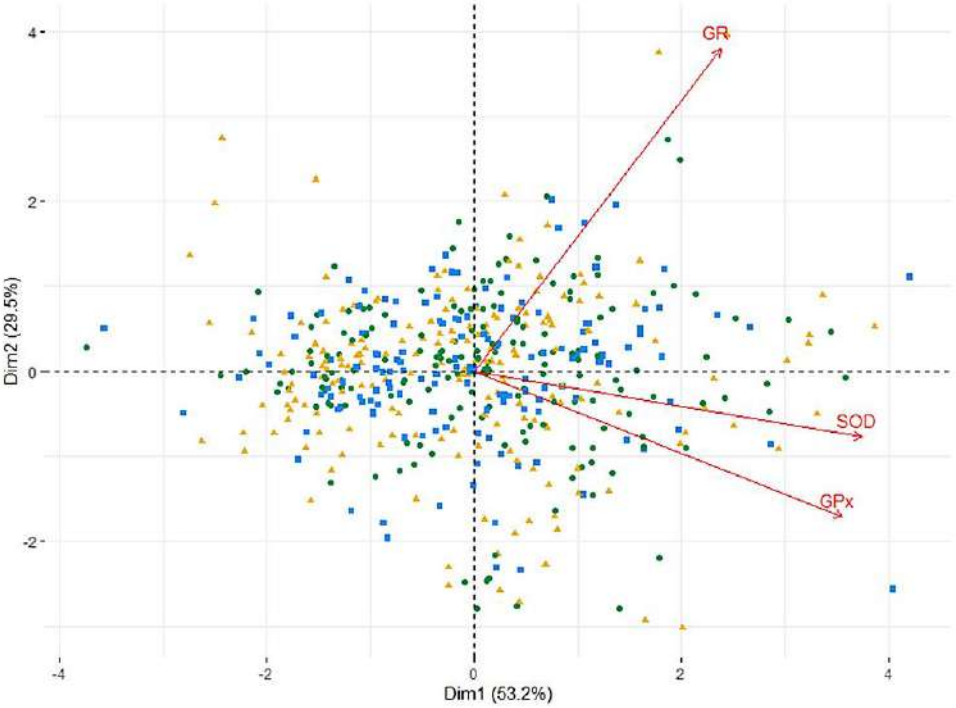


Figure 2. Principal Components Analyses for GPx, GR and SOD activities of juvenile male house sparrows from natural (green circles), rural (yellow triangles) and urban (blue squares) habitats.

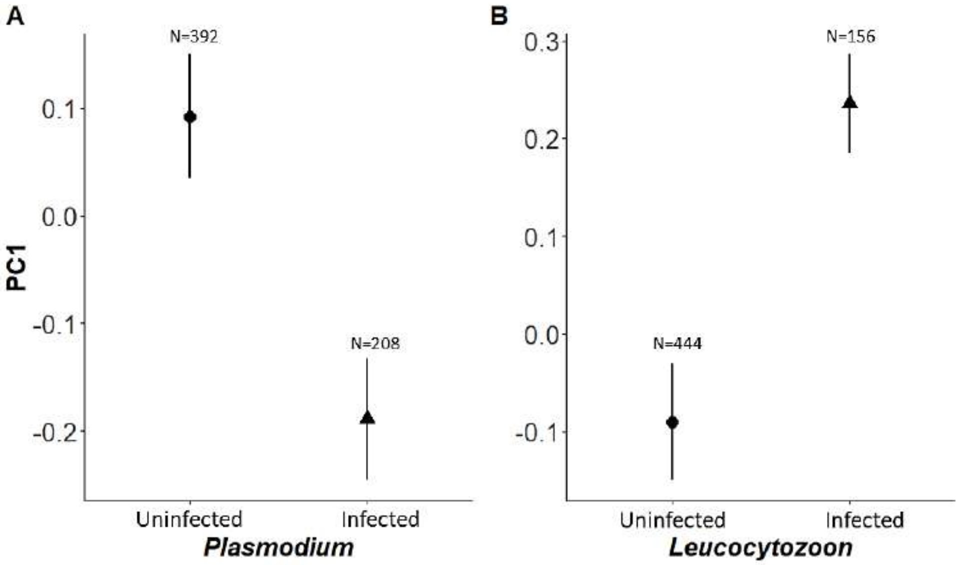


Figure 3. Mean values (± S.E.) of PC1 with respect to bird infection status by *Plasmodium* (A) and *Leucocytozoon* (B). Significant differences were only found for the case of *Plasmodium*.

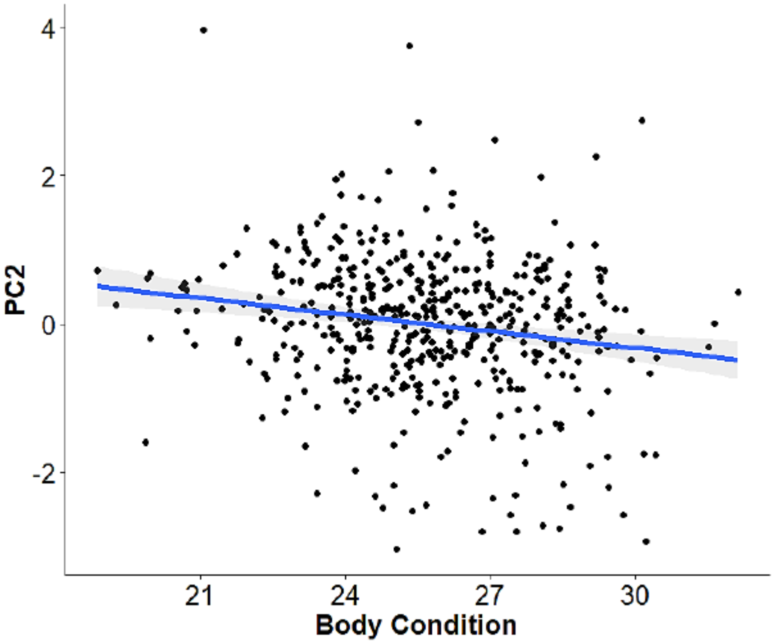


Figure 4. Relationship between PC2 and the body condition of juvenile male house sparrows.

Discussion

We tested the potential effects of major sources of stress for wild birds: urbanization, parasite infections and their interaction. We measured the levels of four different oxidative stress related biomarkers, which reflected the physiological oxidative status of wild juvenile male house sparrows. Our main results showed that the oxidative damage to lipids (reflected in TBARS levels) was higher in birds from urbanized habitats. Also, the relation of TBARS levels with bird body condition was positive in birds from rural and natural habitats but negative in birds from urban habitats. Moreover, the PC1, which reflected the overall enzymatic activity of GPx, SOD and GR, was lower in *Plasmodium* infected birds than in uninfected ones. The PC2, which reflected the relative activity of GR (positive) versus SOD and GPx (negative), was negatively related to bird body condition suggesting that individuals in better body condition had higher activity of GPx and SOD but lower activity of GR.

Effects of parasite infections on the oxidative status of birds

Parasite infections produce deleterious effects on their host by directly draining resources from them and, indirectly, by the cost derived from producing inflammatory and immunological responses to fight this infection. Birds experimentally infected with avian malaria have shown an increase expression level of genes related to both immune and antioxidant responses (Videvall et al., 2015). Indeed, the increased antioxidant activity is probably an attempt to counteract the inflammatory responses and the ROS production against parasites (Sorci et al., 2013; Sorci and Faivre, 2009). However, other stressors or physiological situations may reduce the capacity of birds to mount antioxidant responses involved in the catalytic reactions to remove ROS and avoid damaging effects of *Plasmodium* infection (Isaksson, 2015). For instance, van de Crommenacker et al. (2012) reported an increase in oxidant levels but a decrease in the antioxidant capacity in birds infected with avian malaria parasites during reproduction. Similarly, we found a lower antioxidant enzymatic activity in

Plasmodium infected birds (being reflected in the PC1 component) than in uninfected ones, even we did not find any association between oxidative damage and parasite infection. Our results may indicate that the infection status affects the activity of the redox system of birds, likely as a result of parasite activity or as a response to parasite presence. Immune responses to pathogens may be energetically costly (Martin et al., 2003) so, under natural conditions where available resources are limited, a trade off may exist between developing antioxidant or immune responses (Morales et al., 2006). Curiously, contrary to the case of *Plasmodium*, birds infected with *Leucocytozoon* tended to have a higher GPx, SOD and GR activity (PC1) than uninfected birds. These contradictory patterns found for *Plasmodium* and *Leucocytozoon* could be due to the different infection patterns between parasites (e.g., target infected cells, virulence of each genus) or to the physiological pathways involved in the responses of birds against infections (Valkiūnas, 2005). Indeed, virulence can vary even within diverse parasite lineages of the same genus affecting differently the oxidative stress of birds (Isaksson et al., 2013; Lachish et al., 2011). Nevertheless, in the case of *Leucocytozoon* the relationship with the antioxidant enzyme activity was only shown as a tendency, thus highlighting the need of further research.

Effects of habitat anthropization on the oxidative status of birds

Urbanization processes are largely transforming the landscape and altering the biotic and abiotic factors with implications in life-history traits of several avian species (Bradley and Altizer, 2007; Grimm et al., 2000; Sepp et al., 2018; Sol et al., 2014). Salmón et al., (2018) showed that the urbanization index highly correlates with NO_x levels, one of the main pro-oxidants and stressors substances from urban areas. Pollutants such as toxic chemicals, heavy metal particles and other substances derived from human activities, have negative consequences on urban-dwelling wildlife, especially affecting their health by increasing the oxidative damage and thus causing oxidative stress (Dulisz et al., 2016; Herrera-Dueñas et al., 2014; Isaksson, 2015; Koivula et al., 2011; Meillère et al., 2016; Salmón et al., 2018). Indeed, our results indicated that birds from urban areas showed a higher oxidative damage to lipids than those from rural habitats. Different studies found higher lipid peroxidation through TBARS levels in birds from urban habitats compared to birds from less anthropized ones (Amri et al., 2017; Herrera-Dueñas et al., 2017). Although an up-regulation of antioxidant enzymes under pro-oxidant conditions such as urbanization could be expected (Herrera-Dueñas et al., 2017), we did not find significant differences of the activity of any antioxidant enzymes (i.e., GR, GPx or SOD) in birds from different habitats. Nevertheless, we are aware of the existence of other antioxidant substances not reflected in our indicators such as thiols, carotenoids or vitamins A, C and E, which can be important in the neutralization of ROS by controlling the antioxidant balance of the organisms (Costantini and Verhulst, 2009; Finke et al., 2020; Li et al., 2016; Sorci and Faivre, 2009). Hence, the differences we found could be due to the role of non-enzymatic antioxidants molecules not analysed here.

The relationship between body condition and oxidative status

Our results highlight that the relationship between the levels of TBARS and habitat are influenced by the body condition of birds. Specifically, we found that the oxidative damage to lipids increased with body condition in birds from rural and natural habitats but decreased in urban birds. Besides, we found that PC2, which reflect the relative relationship between the levels of GR (positive) respect to GPx and SOD (negative), decreased as the body condition of the birds increased. In other words, birds with higher body condition had lower GR and/or higher GPx and SOD activities. It is known that the activation of the redox system is an energetically costly process that plays a physiological key role in enabling birds to cope with anthropogenic conditions (Isaksson, 2015, 2020). Under increasing harmful environmental stressors, it is likely that body condition largely determines the capacity of birds to control oxidative stress. Nevertheless, the causality of this effect could not be assessed based on our correlative results; hence, experimental studies would help to further understand the role of the body condition in the capacity of urban dwelling species facing stressors from an anthropic origin.

To sum up, the analysed biomarkers are part of highly integrated physiological responses of organisms that can vary across tissues, host life stages and life history, and thus difficult its interpretation in the wild (Costantini and Verhulst, 2009; Isaksson, 2020). Our results support that the oxidative status of the widespread house sparrows was largely affected by both urbanization and parasite infection. Overall, we show a link among body condition, oxidative damage and the activity of enzymes involved in the redox system, and that parasite infection influenced the antioxidant enzymatic activity of birds. Determining how urbanization affect bird health and what we can do to improve their physiological responses, should be a future goal in conservation biology to counteract the negative effects of urbanization in order to mitigate the population decline of urban house sparrows, among others.

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General section

Discussion

Under the current global change scenario, there is an urgency to identify the dynamics transmission of vector-borne pathogens and to understand how wildlife thrive in anthropogenic altered environments, for both public health and wildlife conservation interest. Both urbanization and infection by avian malaria, common avian vector-borne pathogens, have been pointed out as potential causes of the disappearance of the house sparrow during the last decades. Here, I provide new information on how different anthropogenic landscape uses affects the transmission dynamics of the avian malaria parasites and related haemosporidians, and how both urbanization and parasite infections, affect the health of wild house sparrows along an urbanization gradient, considering urban, rural and natural habitats.

Does habitat anthropization affect the transmission of avian blood parasites?

In **Section 1**, I investigated the interannual differences in parasite prevalence and lineage richness, two major variables reflecting parasite infections dynamics in birds, in house sparrows from habitats with different level of urbanization. Previously, a number of researchers have reported differences between years in the prevalence of blood parasites in birds (Bensch et al., 2007; Dubiec et al., 2016; Geue and Partecke, 2008; Knowles et al., 2011), but results from **Chapter 1** add novel information about the potential differences between habitats with distinct levels of anthropization. The prevalence of both *Haemoproteus* and *Leucocytozoon* parasites differed between years but was also positively correlated between years. No significant differences were found for *Plasmodium* parasites. The prevalence of vector-borne parasites is strongly determined by the abundance and distribution of vectors in the area (Lalubin et al., 2013; Martínez-de la Puente et al., 2013). Differences in the environmental requirements between vector groups (i.e., running water, wet soil or stagnant water), together with habitat characteristics and climatic factors such as temperature and precipitations, strongly determine vector populations (Patz et al., 2008). Moreover, weather conditions greatly affect the activity of blood-sucking arthropods feeding on birds (Martínez-de la Puente et al., 2009a; Merino and Potti, 1996), potentially affecting their exposure to haemosporidians and hence, the prevalence of these parasites in the population. The results of this chapter suggest that populations of both biting midges and blackflies, the main vectors of *Haemoproteus* and *Leucocytozoon*, respectively, may found more stable environmental conditions for their reproduction (e.g., breeding sites) along localities, resulting in the positive correlations in the prevalence between years. Similarly, the annual differences in climate can influence the stability of these breeding sites, explaining then the interannual prevalence differences. Specifically, the interannual differences of *Leucocytozoon* prevalence were found in birds from urban and natural habitats but not from rural habitats. Moreover, in 2013, higher prevalence of this genus was found in natural than in rural habitats. These results are likely explained by the availability among habitats and the stability across time, of running water bodies and thus, breeding areas of blackflies vectors. Lineage richness differed according to habitat type and time. *Haemoproteus* showed, for one-year, higher richness in rural than in urban or natural habitats. Additionally, *Leucocytozoon* richness was negatively correlated between years in rural habitats but positively correlated between years in urban and natural habitats. Our results could be explained by the composition variation of vector (biting midges and black flies) and/or bird communities, which may affect the number and identity of the parasite lineages circulating and infecting house sparrows (Ellis et al., 2020; Ferraguti et al., 2018; Lachish et al., 2013; Ricklefs et al., 2005, 2004; Sehgal, 2010; Wood et al., 2007).

Despite these results could be explained based on the effects that environmental conditions and habitat characteristics can have on the insect vectors of these blood parasites, we cannot rule out the possibility that host-related factors may also explain the patterns of infections found. Indeed, the size, structure and composition of a bird community are highly influenced by landscape alterations and anthropogenic provision of food (i.e., fodder grain for husbandry animals in our study) (Clark et al., 2018; Evans et al., 2009; Shochat et al., 2006). In addition to the effects on host and vector communities, urbanization can also alter the susceptibility of vertebrates to infections through its effects on host immunocompetence (Bradley and Altizer, 2007; Calegaro-Marques and Amato, 2014; Ferraguti et al., 2018; Ishtiaq, 2021; Jiménez-Peñuela et al., 2021; Muriel et al., 2021; Patz et al., 2008).

Does habitat anthropization and blood parasites infection affect birds health?

Environmental and external conditions could determine host ability to modulate immune responses against infections as well as the potential costs of these responses (Arriero et al., 2008; Delhaye et al., 2018b; Morales et al., 2006). It is difficult to isolate the effects of disease from those of food-shortages, external stressors or other environmental conditions that may favour disease transmission or modify the trade-off between birds tolerance or resistance to infections (Arriero et al., 2018; Newton, 1998). For this reason, the next step in this thesis was to measure how urbanization, blood parasite infections and its interaction, was related to the health of wild birds (**Section 2**). For that purpose, I used three different and complementary approximations including body condition (**Ch. 2**) and different eco-physiological markers such as fatty acids composition (**Ch. 3**) and birds oxidative status (**Ch. 4**).

Urbanization effects on birds health

Anthropic activities and habitat alteration had important consequences on the health of wild birds. Our results showed that in urban habitats, juvenile house sparrows had lower body condition (**Ch. 2**), higher quantity of fatty acids (**Ch. 3**), and higher levels of oxidative damage to lipids (measured by TBARS) (**Ch. 4**) than birds from rural habitats. Moreover, when the levels of the urbanization index increased, there was a reduction in body condition in both adults and juveniles

(**Ch. 2**). Additionally, juvenile birds with better body condition had higher activity of GPx and SOD but lower activity of GR (**Ch. 4**).

Some authors have proposed that under a scenario where food sources are abundant and seasonally stable, like urban areas (Shochat et al., 2006), birds do not need to accumulate energy reserves in their bodies, thus presenting low body condition (Salleh Hudin et al., 2016). The results from this thesis, suggest that birds consume more fat-rich food in urban habitats (**Ch. 3**), which is usually related with low concentrations of other essential nutrients (Heiss et al., 2009; Mckinney, 2002; Meyrier et al., 2017). Indeed, food quality and availability are especially important during early life stages to fulfil physiological requirements during development. Nutrient rich food could allow yearlings and juveniles to grow faster, obtain a higher body condition, better immunocompetence and better antioxidant capacity (Chamberlain et al., 2009; Peach et al., 2018; Salmón et al., 2018; Seress et al., 2020; Twining et al., 2018, 2016b). Certainly, our results show that juvenile house sparrows from urban habitats, together with lower body condition (**Ch. 2**) and higher content of fats in plasma (**Ch. 3**), also had higher oxidative damage compared to birds from rural habitats (**Ch. 4**).

Salmón et al., (2018) showed that urbanization levels highly correlate with NO_x levels, one of the main pro-oxidants and stressors substances from urban areas. The high concentration of pollutants such as toxic chemicals, heavy metal particles and other substances derived from human activities, make necessary a strong antioxidant machinery, which has been shown to be upregulated in urban-dwelling wildlife (Dulisz et al., 2016; Herrera-Dueñas et al., 2014; Isaksson, 2015; Koivula et al., 2011; Meillère et al., 2016; Salmón et al., 2018, 2016). The activation of the redox system to respond against urban stressors (e.g., synthesis of HSPs or antioxidant enzymes like GR, GPx or SOD) is energetically costly and could be reflected in a reduced body condition, or if the homeostasis is not correctly maintained, in an increase of oxidative damage (Chávez-Zichinelli et al., 2010; Herrera-Dueñas et al., 2014; Isaksson, 2020, 2015). Different studies found higher oxidative damage to lipids (reflected in TBARS levels) in birds from urban habitats compared to birds from less anthropized habitats (Amri et al., 2017; Herrera-Dueñas et al., 2017). Additionally, our results suggest that urbanization has a large impact in the capacity of birds to dealt with oxidative stress because bird body condition was positively related to TBARS in rural and natural areas but negatively related in urban habitats (**Ch. 4**).

Parasite infections effects on birds health

Parasite infections produce deleterious effects on their avian hosts both by directly draining resources from them and, indirectly, by the cost of inflammatory, immunological and antioxidant responses. Our results shows that juvenile house sparrows infected by *Plasmodium* had less proportion of ω-6 and more of ω-3 PUFAs (**Ch. 3**), as well as lower antioxidant enzymes activity (i.e., GPx, SOD and GR) (**Ch. 4**). Our results are in line with those from van de Crommenacker et al. (2012) who found a low antioxidant capacity in birds infected with blood parasites. During infection, ω-6 PUFA are metabolized to pro-inflammatory prostaglandins, while the down-stream immune components of the ω-3 PUFA pathway is anti-inflammatory (Alagawany et al., 2019; Chang et al., 2018; Hulbert et al., 2005; Simopoulos, 2011). Under natural conditions where available resources are limited, a trade off may exist between developing antioxidant or immunological responses (Morales et al., 2006). Moreover, an excessive pro-inflammatory response, if it is not correctly counteracted by the redox system, can lead to an increment of oxidative stress (Isaksson et al., 2017). *Plasmodium* infections may reduce the capacity of birds to mount antioxidant responses involved in the catalytic reactions to reduce ROS and leading into oxidative damage and oxidative stress (Isaksson, 2015). Thus, our results could be reflecting that, due to the low antioxidant activity in infected birds, exclusively those birds with moderate levels of pro-inflammatory FAs against parasite infection are likely to survive.

Synergic effects of urbanization and parasite infection on birds health

Interestingly, part of the impacts of parasite infections in the health of house sparrows found in this thesis, differed between habitats with different levels of anthropization. Our results support that those birds exposed to two different sources of stress, as are urbanization and parasite infections, may suffer larger deleterious effects. Birds from natural habitats infected by *Plasmodium* showed lower ω-6/ω-3 ratio and tended to have a higher proportion of ω-3 (**Ch. 3**). Thus, it is likely that to promote a higher anti-inflammatory immune response towards *Plasmodium* infections, infected house sparrows preferentially consumed food rich in ω-3 PUFA or mobilize it from storage. This probably happen in natural habitats but not in more anthropized areas due to a differential availability of ω-3 PUFA between habitats (Andersson et al., 2015; Isaksson et al., 2017). Birds with access to better nutritional food has been shown to mount a higher immune response and greater membrane resistance during *Plasmodium* infection (Delhaye et al., 2018a). Alternatively, the high ω-3 and low ω-6 proportion could indicate that less pro-oxidants (originated from ω-6 PUFAs) are being produced to attack the parasites, making birds more susceptible to infections. Moreover, it is also possible that birds from more anthropized environments already metabolized the ω-3 PUFAs to anti-inflammatory components for other reasons (e.g., environmental stressors such as chemicals, pollutants, excessive noise, artificial light, heavy metal particles…) leaving less circulating ω-3 PUFAs to other important functions. Certainly, the presence of ω-3 PUFAs is beneficial during active inflammation to improve the immune response without increasing the oxidative stress (Alagawany et al., 2019).

Contrary to the case of the physiological markers analysed, as well as contrary to what we could initially expect, a higher body condition of juvenile house sparrows was associated with infections by *Plasmodium* and *Haemoproteus* (**Ch. 1** and **2**), which was mainly driven by the data of birds from urban habitats (**Ch.2**). If only infected birds showing a higher body condition are able to face and survive parasite infections (Houston et al., 2007; Moreno-Rueda, 2011; Navarro et al., 2003), it is possible that, due to a differential mortality rate, infected individuals had higher body condition than uninfected birds (Sorci et al., 2013). Moreover, juvenile birds are immunologically naive (Merino, 2010) and could suffer a higher impact of parasite infections than adults. The immunological responses of urban birds to infection are energetically more costly than those of birds from rural habitats, possibly to the limited availability and quality of resources in anthropic areas (Fokidis et al., 2008). For example, great tits from urban habitats had higher expression of immunoregulatory genes than those from natural environments (Watson et al., 2017). This can cause a decrease in haptoglobin production and a loss of body condition increasing the difficulties to survive in anthropic environment (Bailly et al., 2016). Thus, living in anthropized habitats and being infected by blood parasites may impose higher costs due to the exposure to multiple stressors and limited availability of resources. This could be the reason why we find further deleterious effects of blood parasite infections in urban habitats, but not as strong as in rural habitats, where urbanization was moderate, or in natural habitats, which likely have lower presence of anthropic stressors.

Limitations of the thesis and future perspectives

This thesis focuses on three widespread blood parasites infecting birds. However, wild birds, including house sparrows, usually harbour a great diversity of pathogens such as filarial worms and viruses (i.e., West Nile and Usutu viruses) which circulation is known to occur in the studied area (Ferraguti et al., 2020, 2016b; Martínez-de la Puente et al., 2018; Martínez-de la Puente et al., 2019; Roiz et al., 2019). These pathogens are expected to also affect the physiological responses of wild birds, including those studied in the **Section 2** of this thesis. Moreover, this thesis focuses exclusively on a single host species, but the effects of urbanization on physiological traits could differ according to the species analysed (Isaksson et al., 2017). Finally, according to the results obtained in **Section 1**, parasite infections and lineages circulating in the area may vary between years and potentially affect the physiological effects of parasite infections in birds, including those reported in the **Section 2**. In sum, including different species of birds and pathogens, through a longitudinal dataset, may ideally improve the conclusions obtained. However, this is a difficult task especially considering the large sample size required for these studies and the need to study host-parasite interactions in different species across areas differing in landscape use. Nevertheless, although specialist host-parasite assemblages can occur in the studied community, most of the lineages found were not restricted uniquely to house sparrows, probably reflecting a general pattern also in other bird species present in the area. In addition, the identification of parasite impacts on hosts physiology often requires from experimental approaches. In line with our results, a possible experimental reduction of haemosporidian infections in birds from habitats with different levels of anthropization should result in different estimates of the costs of parasitism in each habitat. Nevertheless, we could expect a higher impact on survival and host physiology in birds from urban than in birds from natural habitats.

Despite the identified potential limitations, the results reported here provide novel information on the joint effects of urbanization and parasite infections in wild birds contributing to the progress in the research area of *Urban Ecology*. In addition, this information could be used in public health projects involving zoonotic diseases affecting birds and humans and/or domestic animals (i.e., West Nile virus) as well as in projects of conservation biology with the objective of promote wildlife conservation in cities. Among other, we highlight the importance of including in the studies of urbanization habitats with different anthropic use like rural habitats, where the effect of human activities to wildlife can differ from cities. These differences can be mediated by the presence of domestic animals influencing pathogen transmission, the availability of suitable breeding areas for vectors as well as resources like food or water than affect the community composition of vectors and hosts. Moreover, it is important to include a variety of specific habitat type, as the effect of urbanization can vary with the characteristics of the locality itself as reported in Box 1. Cities traits can influence the level of pollutants, availability of green areas or the stability of resources, among others, which can determine the final effects of wildlife health and the interpretation that researchers can do from it. The structure and characteristics of cities as well as the surrounding landscapes, widely differ across the globe and has to be taken into account in this kind of studies. Lastly, the recent development of molecular approaches like the study of gene expression together with experiments, which can include modifications of parasite load through medication or adjust the levels of possible stressors from urban areas (i.e., food type, pollutants, light, noise…), may allow researchers to go deeper on the study of urbanization and vector-borne diseases. This would help to better understand the effects that both urbanization and infection have on the ecology and evolution of wildlife under the current global change scenario.

Conclusions

1. The prevalence and lineage richness of two of the three blood parasite genera studied (*Haemoproteus* and *Leucocytozoon*), differed between years and between habitats with different levels of anthropization. Thus, the different patterns of variation in the infection by *Plasmodium*, *Haemoproteus* and *Leucocytozoon*, outline the importance of considering the specificities of each parasite-insect vector system, especially at human-altered environments.

2. Both urbanization and parasite infection were related with the body condition of house sparrows, with juveniles infected by *Plasmodium* or *Haemoproteus* from urban habitats, showing a higher body condition. This could be due to the selective mortality of those individuals with lower body condition unable to thrive with the negative joint effects of both urbanization and parasite infection.

3. The composition of plasma fatty acids of juvenile house sparrows differed with urbanization and infection status. The relative proportion of ω-6 and ω-3 PUFAs differed between *Plasmodium* infected and uninfected birds. Moreover, in natural habitats, the ω-6/ω-3 ratio was lower in infected than uninfected birds. This suggests that habitat may influence the composition of PUFA and thus, affect the capacity of individuals of mounting an anti-inflammatory or pro-inflammatory response against *Plasmodium* infections. Consequently, habitat anthropization may affect the birds capacity to fight parasite infections.

4. The oxidative damage to lipids suffered by juvenile house sparrows was larger in urban than in rural habitats. The oxidative damage increased with bird body condition in rural and natural but decreased in urban habitats. This highlight that urban dwelling birds may be exposed to physiological stress and be less able to cope with it, as suggested by the negative relationship between oxidative damage and body condition in these areas.

5. The antioxidant activity in juvenile house sparrows varied with blood parasite infection and the body condition of birds. Birds infected by *Plasmodium* had higher antioxidant enzyme activity (GPx, SOD and GR) than uninfected birds. Moreover, birds with higher body condition had higher activity of GPx and SOD but lower activity of GR enzyme. These suggest that blood parasite infections affect the correct maintenance of the oxidative status of birds and that bird body condition is related to the function of the antioxidant system.

6. Landscape anthropization affects parasite transmission and more importantly, determine the impact that parasite infection may have on host health. The results of this thesis emphasise the synergistic effects that anthropization may have in combination with other stressors such as parasitism. The alteration of parasite transmission dynamics and its increased impact on host health in human-altered environments may have important consequences for the conservation of biodiversity.

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Valencia, Junio 2016. Estaba terminando las dos últimas asignaturas del grado, colaborando donde hice el TFG y estudiando para sacarme el B1 de inglés. ¿Y ahora qué? Sabía que quería hacer un máster pero quedarme en la Universidad de Valencia sentía que se me quedaba pequeño. Por tanto, mi otra opción era salir de casa por primera vez, vivir lejos de familia y amigos, conocer otra ciudad… Me inundaban las ganas y el miedo a partes iguales… menos mal que ganaron las ganas. Lo siguiente era ganar dinero. Gracias a una amiga empecé a trabajar en un restaurante donde me quedé todo el verano. En una de mis tardes de búsqueda apareció un máster en Sevilla sobre biodiversidad y conservación, con convenio con la Estación Biológica de Doñana. "PFF DOÑANA". Y pensé "mira, yo no sé si el máster me gustará, pero la aventura me la llevo seguro". El día que me dijeron que entraba en el máster nadie del turno se quedó sin saber que había entrado. "¡Que me voy a Sevilla!". Sabía que iba a ser una experiencia espectacular, lo que no tenía ni idea era de cómo me cambiaría la vida gracias a esta decisión.

Sevilla, Noviembre 2016. Llegué un fin de semana y cuando fui a dar mi primer paseo por el cauce del Guadalquivir todo el mundo iba *de sevillanas maneras.* "No voy a encajar aquí en la vida", pensé. Pero en el máster tuve la suerte de encontrarme con una combinación de gente espectacular que iba a hacer que los siguientes 9 meses fueran de los mejores de mi vida. Después fue cuando llegó mi golpe de suerte y mi mejor causalidad en mi camino como bióloga. Había que elegir TFM. Yo venía de limnología, la cual disfruté mucho, pero sabía que no era lo que quería hacer. La respuesta la había tenido toda la vida delante: aves. Hice varias entrevistas con los investigadores que ofrecían proyectos, pero hubo uno que lo cambió todo. "Gorriones, malaria aviar y urbanización". Y ahí estaban ellos: Josué y Martina. Me contaron el proyecto y su forma de trabajar, y cuando salí pensé "no puede ser tan perfecto". Llamé a mi madre y le dije "mamá, ojalá me cojan, porque me encantan". Martina siempre me dice que le impresionó mi *pelazo* y eso me dio puntos para que me eligieran. Menos mal que *"tengo pelazo y cerebro debajo"* (Vecina rubia, 2019). Así que empecé mi camino con los Figuerólidos&CIA: Jordi, Josué, Martina como mis directores y Rafa, Alazne, Ramón, Alberto, Isa, Lauras, Óscar, Nene, Jiayue... como compañeros. Trabajar con ellos fue un auténtico placer. Jamás me había sentido tan cómoda trabajando en un equipo. Desde el primer momento me integraron como una más. Fueron impulso. Estaban dispuestos a escucharme, guiarme, ayudarme, enseñarme y a meterse mucho conmigo por ser la pequeña, por supuesto, porque si no, no serían los Figuerólidos. Yo que había llegado al máster sin ninguna idea de qué camino quería seguir, se preocuparon por mi formación, por mi experiencia, por que conociera todo lo que suponía investigar. ¿Investigar? Yo venía a Sevilla a ver qué pasaba… ¿Y qué pasó? La buena ciencia. Fui una de las primeras de clase en defender mi TFM, disfruté como una niña pequeña del proceso y quedé super contenta. "Yo me quiero quedar aquí, con ellos". ¿Doctorado? ¡Doctorado!

Valencia, Julio 2017. Ese verano volví al restaurante a trabajar. Tenía que buscar becas de doctorado y pensar en un posible proyecto. Los primeros bocetos, dudas e ideas de esta tesis los tengo escritos en la parte de atrás de tickets del restaurante. En septiembre apareció una primera oportunidad con la Fundación Tatiana Pérez de Guzmán el Bueno… ¿Probamos? ¡Probamos! La beca era sólo de tres años así que lo más sensato era aprovechar todas las muestras que se habían recogido durante la tesis de Martina, con las que había muchísimas posibilidades. Debo admitir que al principio estaba un poco triste, porque yo quería un proyecto grande, propio, con mucho trabajo de campo y hacer muchísimas cosas (lo típico). Pensaba que todo eso no podría hacerlo, pero qué equivocada estaba (¡Novata!). Presentamos el proyecto y fue seleccionado. Después de una entrevista en Madrid, el 13 de Noviembre me llegó el mensaje que te da la vuelta al corazón: *Nos alegra comunicarle que el Patronato de esta Fundación, ha resuelto favorablemente su solicitud de Beca Predoctoral en Medioambiente - 2017.* ¡TENÍA DOCTORADO! Me sentí (y me sigo sintiendo) profundamente afortunada por conseguir la primera beca que pedí y poder hacer mi tesis donde quería, con quien quería y con un tema que me apasionaba.

Sevilla, Diciembre 2017. Fue el inicio de mi camino como doctoranda y Figuerólida oficial… hasta hoy. ¡Menudo privilegio! A mis Figuerólidos les debo todo en lo que me he convertido. En primer lugar, **a mis directores**. Los mejores que podía tener. Yo sé que no es fácil lidiar conmigo. Soy curiosa, preguntona y cabezona, sobre todo cabezona. Quiero saberlo y entenderlo todo. Nunca me ha servido que me digan "esto es así y punto". Necesito comprenderlo y hacerlo mío, y no paro hasta que lo consigo. Vosotros siempre habéis estado ahí para mí, con disponibilidad y paciencia. Habéis respetado mis ritmos y mi forma de trabajar. Y por eso sólo puedo deciros gracias, gracias y gracias. He crecido muchísimo de vuestra mano y sólo espero seguir haciéndolo durante los próximos años.

Jordi. Tenemos mucha suerte de tenerte. Cada día me sigue impresionando tus capacidades para poder lidiar con todo, protegiéndonos a todo el grupo bajo tus alas para poder salir adelante y preocupándote por cada uno de nosotros. Has estado siempre disponible para cualquier duda o pregunta, dispuesto a debatir conmigo y con una respuesta correcta para todo. Eres claro, preciso, conciso y directo (¡para bien y para mal!). Has tenido muchos puntazos durante las eternas revisiones de manuscritos, pero algunos de mis comentarios preferidos han sido "*¡¡Este término que usas hace que me salgan sarpullidos y mueran delfines en el mar!!*" o *"Future wildlife? Esto qué es ¿La evolución de los Pokémon?"*. Prometo por el bien de tu piel y de los pobres delfines mejorar en el uso de ciertos términos, y por supuesto, no juntar nunca más en un mismo modelo hábitat e índice de urbanización JAJAJA. Espero que además de mi crecimiento científico, también hayas quedado contento con mi creciente habilidad culinaria para las paellas valencianas. Las comidas en tu casa y los baños en la piscina han hecho más por el bienestar mental del grupo de lo que jamás imaginarías… ¡Espero que nunca se pierdan y poder asistir a muchas más!

Josué. Mil gracias por todo lo que has hecho por mi durante estos años. Tienes un corazón que no te cabe en el pecho. Tanto a nivel profesional como personal eres una de las personas más maravillosas que he conocido en mi vida. Gracias por guiarme, por enseñarme, por protegerme y por mostrarme la cara más bonita y pasional de la ciencia. Gracias por tu paciencia, por tu cariño, disposición, paciencia (otra vez) y por tu humor. Contigo he aprendido muchísimo y me he reído hasta llorar de la risa a partes iguales, y eso no tiene precio. Te mereces todo lo bonito que el universo te tenga preparado, tanto en la ciencia como en la vida. Puedes estar muy orgulloso de quién eres y de cómo te construyes cada día (y nos construyes a los demás). Espero poder seguir disfrutando de nuestras charlas y de tus consejos durante muchos años más… ¡Esto no me lo pierdo! Además, me alegra haber encontrado a alguien que también parece super enfadado cuando solo está concentrado. Y por supuesto, agradecerte la lección más importante en estos años… “Con los dedos de la mano, los dedos de los pies…” ¡Y menos mal que mi tesis sólo tiene cuatro capítulos! ¡POR UNO! ¡De la que me he librado…!

Martina. Dicen que la perfección no existe, pero la verdad es que tú, te acercas mucho a ella. Has confiado en mí desde el primer momento. Siempre has estado dispuesta para cualquier cosa que he necesitado, tanto estando a metro y medio como a kilómetros de mí, y siempre con una sonrisa. Eres unos de mis primeros referentes femeninos en esto de la ciencia. Me has enseñado que el camino no es fácil, pero que el trabajo y el esfuerzo continuo ayudan a avanzar. Que la actitud, tanto en la ciencia como en la vida, lo es todo. Y el *brillibrilli* y los colores, indispensables. Durante estos años hemos crecido juntas, y quiero que sepas que estoy muy orgullosa de ti. No sé si yo habré sido una buena doctoranda durante este tiempo, pero te aseguro que tú, has sido una muy buena directora. Tener a alguien cerca que acababa de pasar por todo el recorrido me ha ayudado más de lo que imaginas. Gracias por guiarme, ayudarme siempre y, sobre todo, gracias por contar conmigo. Es todo un honor para mí que quieras mantenerme cerquita, y no dudes en ningún momento, que lo estaré. Nos esperan cosas muy chulas juntas (¡Y nadie podrá evitar que pongamos las gráficas con colorinchiiiis! muhahahaha).

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Ramón. Siempre tienes las palabras perfectas para el momento justo. Y siempre son cosas bonitas de escuchar. Muchas gracias por ayudarme y guiarme, y por arroparme como una más del grupo desde el primer momento. Doñana vive en ti, no hay ninguna duda de eso. Gracias por todas las cosas pequeñas y bonitas que nos enseñas. Y aprender a disfrutar de lo que trata la vida: algo tan sencillo como un cielo estrellado, un paseo, una buena comida o un ratito charlando… Lo más importante es la compañía y la tuya es inmejorable.

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Laura, Jiayue, Wouter... Ha sido un placer haberos tenido en el grupo y aprender muchísimas cosas con vosotros. **Isa** y **Laura**, el motor por excelencia del laboratorio. Gracias por estar siempre para enseñar y ayudar, grandes trabajadoras natas de las que aprender constantemente. Gracias por vuestra paciencia y gracias Isa, por tratarnos siempre como a tus pequeños. **Juan y Olaya,** últimas incorporaciones que estoy segura de que van a llegar muy lejos. Me da penita no veros crecer al pie de cañón, pero ha sido todo un placer compartir ratitos en el campo y en el despacho con vosotros y sólo espero que nos queden muchos más. **Nene**, gracias por tu locura y tu actitud que siempre animan incluso en esos días más largos y pesados. Si la gente fuera un poco más como tú, el mundo sería más feliz. Y por supuesto gracias por estar siempre dispuesto a enseñar con paciencia y cariño. **Santiago, Esme, Juani, Álvaro** y en general a todos los del equipo mosquitos, sois un solete. Me quedo con la espinita de no haber aprendido todo lo que me hubiera gustado de vosotros. Gracias a todo el resto del **personal de la EBD** que con paciencia y cariño han estado dispuestos a ayudarte cuando algo hacía falta, y que han hecho de la EBD una experiencia redonda. Técnicos de laboratorio y de campo, secretaría, limpieza, dirección, informática, recepción, equipo del LAST… Gracias a todos mis **compañeros doctorandos**, que siempre han sido majísimos y se han portado genial conmigo. Es una pena no haber disfrutado al 100% por la pandemia, pero me quedo con pequeños buenos recuerdos.

Lund (Sweden), September – December 2019. During three months of my phD period I was lucky enough to experience an international internship with one the most powerful group in urban ecology. Thank you, ***Johan****. I really appreciate how you take me in the university helping me with all the bureaucracy and lab work. Thank you for your patience while I tried to get those impossible “*snowballs*” and for being available for any question, no matter how many were. Also, I want to thank* ***Hong-Lie*** *for helping me during the more difficulties of the lab work, for your patience and for explaining everything so perfectly. It would have been impossible without your help. Moreover, thank you a lot to all the MEEL team for letting me participate in your meetings and make me feel so comfortable with you all. It was a pleasure. Lastly and most importantly, thank you* ***Caroline*** *for give me the opportunity and making it easy since day one. For being available for me during several months before the internship planning everything, during the internship to solve any doubt, despite the complicated timing, and now after the internship, writing the manuscript. I hope this is our first work together but not the last.* Estos meses además los disfruté en la mejor compañía: **Dani, Javi, Luz y Violeta.** Gracias por hacer de mi estancia un periodo muchísimo más divertido. Por nuestras escapadas para conocer Suecia y nuestras sobremesas particulares. Especial mención a **Dani** por nuestros momentazos en el despacho y a **Violeta** por nuestras escapadas de desconexión para ponernos *pretties* y comer (y beber) mucho. *Lastly, I want to thank the sweetest French girl I could ever met,* ***Samantha****. I was very lucky to have you around and you made my stay even more special with our talks and your restorative hugs.*

Además del doctorado y los Figuerólidos, mi primer año en Sevilla me regaló a unas personas increíbles que me llevo conmigo para el resto de mi vida y que han sido fundamentales durante estos años.

Isa. Cumplimos nuestra promesa de vivir juntas y no sé qué habría sido de mí si no lo hubiéramos conseguido. Has sido, eres y serás mi apoyo incondicional. Quien ha celebrado conmigo cada victoria y cada derrota. Quien ha estado ahí, siempre, para escucharme y darme un abrazo cuando lo necesitaba. Nos hemos mantenido sanas la una a la otra durante una tesis y una pandemia mundial ¡a la vez! Si nos llegamos a complementar mejor reventamos. Maridas se queda corto. No tengo vida (ni hojas de tesis) suficiente para agradecerte todo lo bien que te has portado conmigo y todas las cosas que hemos vivido juntas.

Sara. Te echo muchísimo de menos, pero sólo puedo pensar lo afortunada que he sido de poder disfrutarte (y a Carlos) todos estos años tan cerquita. Has sido un pilar fundamental en mi vida. Los paseos, las charlas, las comidas y las cenas juntos, las risas, las tonterías y por supuesto… tus zascas y tu sinceridad aplastante. Eres impresionante, por dentro y por fuera. Valiente, serena y llena de cariño. La vida tiene muchas cosas bonitas para ti, y yo sólo espero poder verlas a tu lado. Gracias por hacernos partícipe de lo bonito que es crecer una vida. Ya estoy deseando llegar a la isla para comeros a besos.

Jose. Cada vez que nos vemos es posible que acabemos llorando de la risa. Gracias por ser mi fuente continua de momentazos (y rebuznitos). Pajarear, playeo, piscina, paseo, comer churros, un ajedrez… cualquier plan es bueno, y me han dado la vida estos años. Gracias por, dentro de todas estas risas, esos momentos también de conversaciones profundas sobre la vida. Soy muy afortunada de tenerte y poder darnos consejitos. Además, eres el culpable de nuestras altas expectativas con los coches. Estoy segura de que nos quedan muchos viajes con el techo abierto, viendo rapaces y con la música a tope.

Pili. Gracias por los ratitos de (des)conexión y por esa paz y tranquilidad que transmites, aunque por dentro seas un manojito de nervios. Si hay plan para pasar el día en tu paraíso personal sabemos que el día va a salir bien. Gracias por dejarnos pasar tantos buenos ratos allí y por llenarme de amor perruno cuando tengo a la mía lejitos. Gracias por acogerme desde el principio en tu casa como una más y hacerme sentir tan cómoda siempre. Y por supuesto, gracias por esos ratitos de feria al más puro estilo sevillano. Con mil ganas de poder disfrutar de otra más juntas.

David. Has sido mi mejor casualidad. Amigo, amante y compañero. Has estado ahí por y para mí desde el primer momento, creyendo en mí ciegamente. Celebrando todas mis victorias como si fuera lo más increíble del mundo y quitándole hierro al asunto cuando algo salía mal. Estamos superando dos tesis, una pandemia y una relación a distancia… Y contra todo pronóstico aquí seguimos. Cada día más fuertes, queriéndonos más y mejor. Estoy segura de que esto sólo es el principio de todo lo que nos queda por vivir... Y estoy deseando disfrutarlo. No sé por dónde nos llevará el camino de la ciencia y de la vida, pero yo estoy dispuesta averiguarlo si lo hacemos juntos. Porque te quiero, sólo un poco.

También quiero dar las gracias a los mejores amigos que podrían existir, que creen en mí y me apoyan siempre, aunque les hable de cosas raras. Biofuck: **Héctor, Carla, Marina, Sandra, Loren, Ali**; lo más bonito que me ha dado la natación: **Salva, Laura, Bea(s)**; mis pequeños tesoros del instituto: **Cris y Neus**; personitas tan especiales como **Iris** o **Denitsa.** Gracias a cada uno de vosotros por acogerme como si el tiempo no hubiera pasado cada vez que volvía a *la terreta*. No me olvido mis asturianos bonicos **Lara** y **Manu,** por regalarme tantos momentos especiales y estar siempre ahí para mí para disfrutar juntos un día de campo, una quedada en el norte o una cervecita por el sur.

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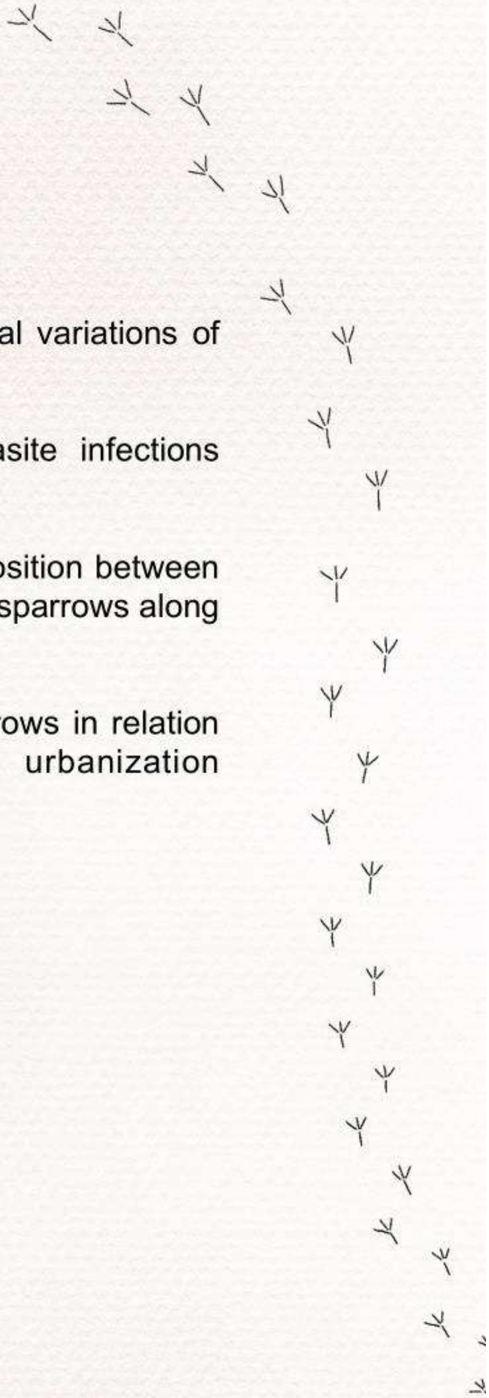
Y por último, por supuesto, agradecer a mi inspiración y mi fuerza. Mi ángel de la guarda. Mi **iaio**. Qué sería de mí, quién sería yo ahora mismo, si no te hubiera tenido a ti. Mi pasión por las aves es fruto de tu pasión. Nacido de uno de los momentos más bonitos de mi infancia: los domingos en casa de los iaios con los tíos y los primos para comer paella todos juntos. Nada más entrar en su casa los escuchabas: Un cuarto lleno de canarios, con un canto precioso. Cuando llegaba la primavera, mi obsesión era ver cómo hacían los nidos, ponían huevos, los incubaban, nacían e iban creciendo los pequeños. Siempre había una silla preparada en la que me podía subir, o tú me cogías en brazos y me aupabas para ver los que estaban más alto. No pudiste llegar si quiera a ver cómo me convertía en bióloga. Pero sé que me cuidas, me ves, me proteges y me das fuerza. Sé que me sonríes desde el arcoíris de los iaios cada vez que cojo un pájaro de la red y que vuelas libre junto a ellos. Te tengo presente cada día de mi vida. Gracias por todo lo que me has enseñado y todo lo que has hecho por mí. Te quiero, iaio.

Gaia.

Por el final del principio.



Chapter 1. Urbanization effects on temporal variations of avian haemosporidian infections.

Chapter 2. Urbanization and blood parasite infections affect the body condition of wild birds.

Chapter 3. Differences in fatty acids composition between *Plasmodium* infected and uninfected house sparrows along an urbanization gradient.

Chapter 4. Oxidative stress in house sparrows in relation to haemosporidian infection along an urbanization gradient.

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